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Genetic diversity, gene flow and inbreeding in pedunculate oak (*Quercus robur* L.) in fragmented forest stands

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Dankwoord

"If I have seen further it is by standing on the shoulders of giants" ~ Isaac Newton

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Guy,
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"He that plants trees loves others besides himself" ~ Thomas Fuller

Summary

Deforestation and fragmentation of once large and continuous forests are probably one of the most important and widespread human-induced changes that have been made to forests worldwide. Small forest fragments will contain small tree populations, which are more prone to extinction and to losses of genetic variation through increased random genetic drift, selfing and mating among closely related individuals. A good understanding of the processes that shape genetic diversity across generations within tree populations is therefore indispensable for the evaluation and conservation of the evolutionary potential of trees in the long term and for increasing individual tree performance in the short term.

Nevertheless, despite the field of forest landscape genetics has been an attractive science domain throughout the past decades, our knowledge of the genetic consequences of anthropogenic disturbances for tree populations remains relatively limited. Furthermore the effects of forest fragmentation and forest management practices on the genetic diversity of tree populations are highly debated in the scientific literature, resulting in what is known as the "paradox of forest fragmentation genetics". This thesis aimed at gaining better insights into the effects of human land use and forest management activities on the genetic diversity, gene flow and inbreeding of small and fragmented pedunculate oak populations (*Quercus robur* L.). To do so, a multidisciplinary approach was adopted, in which we first conducted a meta-analysis of the available literature on forest landscape genetics. Subsequently we examined the maintenance of genetic diversity, contemporary gene flow and individual tree performance in small and fragmented pedunculate oak stands in Northern Belgium.

Based on our meta-analysis, we could reject the traditional idea that woody plant species are relatively resistant to genetic losses following habitat fragmentation, as they were as prone to genetic erosion as herbaceous species studied in earlier meta-analyses. Although wind-pollinated species are presumed to have extensive pollen flow, we found evidence for pollen limitation and genetic losses, which were similar to those observed in insect-pollinated species.

When we compared the results of this meta-analysis with what was found in our in-depth study on pedunculate oak, no clear losses of genetic diversity were observed across generations in the studied pedunculate oak stands. This suggest that the effective population sizes (N_e) were large enough to avoid the negative impacts of genetic drift and inbreeding. Since high out-of-plot pollen immigration rates were observed in all study plots, extensive pollen flow may have counteracted the negative genetic consequence associated with small tree populations. Nevertheless, even under high out-of-plot pollen immigration rates, we found that an important fraction of mating events occurred at short distances between neighbouring trees. Also in our study plots, less diverse local pollen pools were obtained in small and low-density forest stands, which suggested that pollen limitation due to a restricted number of local mating partners was not totally compensated by the observed high out-of-plot pollen immigration rates. Ultimately, this may increase the likelihood of inbreeding in subsequent generations. The heterozygosity level of tree individuals should however be maximized in natural populations, as we detected a weak but significant relationship between heterozygosity and tree performance. Moreover our study was one of the first that showed an effect of high drought stress levels on the strength of the observed heterozygosity-fitness correlations. This suggests that in view of climate change the genetic consequences of forest fragmentation could be further exacerbated.

To mitigate the potential negative genetic consequences through increased inbreeding and to maintain rare, potentially adaptive alleles in small pedunculate oak stands, N_e should be maximized in the seedling cohort. In this respect conservation resources should be directed to establish genetic connectivity across fragmented oak populations. However this is not always realistic in the highly fragmented forest landscapes of Northern Belgium, through which more customized management practices are needed to maintain or increase N_e. Supplementation of natural regeneration by composite provenancing may be a good option to maintain and increase genetic diversity in small and fragmented oak stands and to enlarge the evolutionary potential to current and future environmental changes.

Samenvatting

De ontbossing en versnippering van grote en aaneensluitende bossen zijn één van de meest ingrijpende en wijdverspreide veranderingen geweest die door de mens aangericht werden in bossen overal ter wereld. Kleine gefragmenteerde bossen herbergen kleinere populaties van bomen, dewelke meer kans hebben om lokaal te verdwijnen en om genetische diversiteit te verliezen door een verhoogde genetische drift, zelfbestuiving en inteelt. Een goed wetenschappelijk inzicht in de processen die de genetische diversiteit van generatie op generatie vorm geven is daarom cruciaal voor de evaluatie en het behoud van het evolutionair potentieel van boomsoorten op lange termijn en voor het verhogen van de individuele fitness op korte termijn.

Ondanks dat de landschapsgenetica van bosbomen een aantrekkelijk studie-domein was gedurende de afgelopen decennia, is onze kennis van de genetische consequenties van menselijke verstoringen op bomenpopulaties relatief beperkt. Daarenboven is er in de wetenschappelijke literatuur controverse over het effect van bosfragmentatie en bosbeheer op de genetische diversiteit van bomen. Dit doctoraat heeft als doel een beter inzicht te verkrijgen in de effecten van menselijke landgebruik en bosbeheer op de genetische diversiteit, gene flow en inteelt van kleine en gefragmenteerde zomereikenpopulaties (*Quercus robur* L). Om dit te bewerkstelligen werd voor een multidisciplinaire aanpak gekozen, waarbij we eerst een meta-analyse van de beschikbare literatuur over de landschapsgenetica van bosbomen uitvoerden. Vervolgens onderzochten we de genetische diversiteit, hedendaagse gene flow en de individuele fitness van zaailingen in kleine en gefragmenteerde zomereikenbestanden in Vlaanderen.

Op basis van onze meta-analyse, konden we de traditionele gedachte dat houtige plantensoorten relatief resistent zijn tegen het verlies van genetische diversiteit na habitatfragmentatie verwerpen. Bomen en struiken bleken zo gevoelig voor genetische erosie als kruidachtige soorten bestudeerd in eerdere meta-analyses. Ook vonden we effecten van pollen-limitatie en verlies aan genetische diversiteit bij wind-gepollineerde soorten, die vergelijkbaar waren met de effecten waargenomen bij door insecten bestoven boomsoorten.

In tegenstelling tot de resultaten van onze meta-analyse werd in onze meer diepgaande studie op zomereik geen duidelijk verlies van genetische diversiteit tussen generaties waargenomen. Dit suggereert dat de effectieve populatiegroottes (N_e) van de onderzochte bestanden voldoende groot waren om de negatieve effecten van genetische drift en inteelt te vermijden. Aangezien een aanzienlijk gedeelte van de pollen afkomstig was van buiten de studie plots, konden de negatieve genetische gevolgen geassocieerd met kleine populaties tegengewerkt worden door een omvangrijke pollen flow. Toch vonden we dat ondanks een sterke pollenimmigratie, een belangrijk deel van de bestuivingen plaatsvond op korte afstanden tussen naburige bomen. In de kleinere en lage-densiteits eikenbestanden observeerden we ook een verminderde pollendiversiteit. Dit suggereert dat de pollen-limitatie die veroorzaakt werd door het beperkte aantal lokale reproductieve partners, niet gecompenseerd werd door een sterke pollenimmigratie, wat de kans op inteelt in volgende generaties verhoogt. De heterozygositeit van bomen zou echter behouden moeten blijven in natuurlijk populaties, aangezien we een zwakke maar significante relatie tussen heterozygositeit en individuele fitness terugvonden. Bovendien is onze studie één van de eerste die een effect van droogte stress op de sterkte van de waargenomen heterozygositeits-fitness correlaties kon aantonen. Wat aangeeft dat de gevolgen van genetische erosie in gefragmenteerde eikenpopulaties versterkt zouden kunnen worden door een mogelijke klimaatsverandering.

Om de mogelijke negatieve genetische gevolgen van verhoogde inteelt tegen te gaan, en potentieel adaptieve allelen te behouden, zou N_e gemaximaliseerd moeten worden in de zaailingengeneratie. Met dit als doel, zouden de beschikbare middelen in de eerste plaats aangewend moeten worden om de genetische connectiviteit tussen gefragmenteerde eikenpopulaties te verzekeren. Dit is echter niet altijd realistisch in het sterk gefragmenteerde boslandschap van Vlaanderen, waardoor meer aangepaste beheerspraktijken nodig zijn om N_e te vergroten. Het aanvullen van de natuurlijke verjonging door reproductief materiaal verzameld van lokale en niet-lokale bronnen is een veelbelovende optie om de genetische diversiteit in kleine en gefragmenteerde bestanden te behouden, en om het evolutionair potentieel van bomen te vergroten en ze beter te wapenen tegen huidige en toekomstige omgevingsveranderingen.

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List of abbrevations

A: mean number of alleles per locus

A_{base}: area at base of stem or branches

AFLP: amplification fragment length polymorphism

AIC: Akaike's information criterion

A_n: number of different alleles

A_r: allelic richness, corrected for differences in sample size

A_{top}: area at top of stem or branches

$B_{D,base}$: branch diameter at base

$B_{D,top}$: branch diameter at top

B_L: branch length

B_n: number of branches

CI: confidence interval

C_p: Mallow's C_p selection criterion

CT_i: cumulative transpiration standardized for vapour pressure deficit

d: Hedge's d

d^2: metric to detect HFCs; squared difference between the two alleles at a locus, averaged over all microsatellite loci

D: Nei's unbiased genetic distance between seed families

F_{ij}: Nason's kinship coefficient

F_{IS}: inbreeding coefficient

F_{st}: genetic differentiation between populations

g_s: stomatal conductance

HFC: heterozygosity-fitness correlation

H_e: expected heterozygosity

H_o: observed heterozygosity

L_n: number of leaves

LOD: log-likelihood ratio

MLH: individual multilocus heterozygosity

N_e: effective population size

$N_{e(v)}$: variance effective size within seed families

NGS: next-generation sequencing

P: percentage of polymorphic loci

PAR: photosynthetically active radiation

pF: suction pressure

$Q_{between}$: Cochran's Q: heterogeneity test in meta-analysis

QTL: Quantitative trait loci

R^2_{adj}: R^2 coefficient adjusted for the number of predictor variables in the model

RAPD: random amplified polymorphic DNA

REW: relative extractable soil water

RGR: relative growth rate

RH: relative humidity

r_p: correlated paternity

R^2_p: partial R^2 coefficient

r_s: Spearman rank correlation coefficient

s: selfing rate

$S_{D,base}$: stem diameter at base

$S_{D,top}$: stem diameter at top

SE: standard error

SGS: spatial genetic structure

S_L: stem length

sMd^2: d^2 standardized with its locus-specific variance

SNP: single nucleotide polymorphism

SSR: simple sequence repeats

T_a: air temperature

TE: transpiration efficiency

t_m: multilocus outcrossing rate

TR: transpiration rate

t_s: single-locus-outcrossing rate

VPD: vapour pressure deficit

V_{tot}: total woody volume (stem + branches)

W_{AG}: above-ground biomass

WC: water content

W_D: dry weight

W_F: fresh weight

WP: biomass water productivity

Θ_{xy}: average coancestry coefficient within seed families

θ_{FC}: volumetric soil water content at field capacity

θ_V: volumetric moisture content

θ_{WP}: volumetric soil water content at wilting point

Φ_{FT}: molecular differentiation between global pollen pools

ψ_{md}: midday leaf water potential

ψ_{pd}: predawn leaf water potential

$\Delta\psi$: water potential range

Chapter 1.

General Introduction

1.1 Forests in a changing world

Trees and shrubs are keystone species in many terrestrial ecosystems on earth (Lindenmayer & Franklin 2002). As ecosystem engineers, they determine the architecture and the micro-climate conditions of forests, creating resource niches for numerous other organisms (Wright & Jones 1996; Lonsdale et al. 2008). Moreover, trees and shrubs provide a multitude of ecosystem services beneficial for human life, including climate mitigation, water regulation and biomass production (Myers 1997). Despite their global importance, the functioning and sustainability of forest ecosystems have been increasingly challenged by various anthropogenic threats. Deforestation is one of the most important and widespread human-induced changes that have been made to the Earth's surface (Williams 2002). Both in tropical and temperate regions, increasing demands for food and changes in land use have replaced once large and continuous forests by a mosaic of forest patches embedded in an agricultural or urban matrix (Riitters et al. 2000). Globally, the cumulative loss of forest area over the last 5000 years have been estimated at 1.8 billion hectares (Williams 2002), resulting in a forest cover of about 31 percent (ca. 4 billion hectares) of the earth's land surface today (FAO 2010). During the last decades the rate of forest clearance showed signs of slowing down (Figure 1.1), however losses of forest

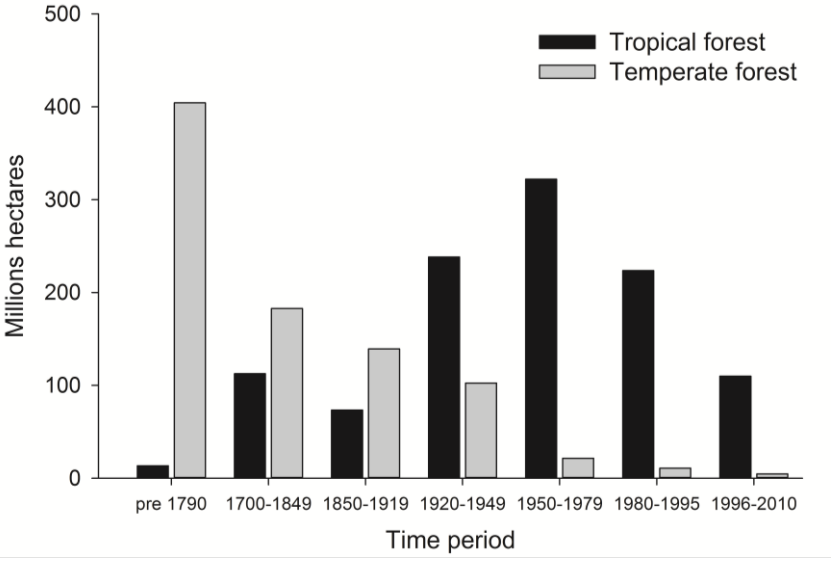

Figure 1.1: Estimated deforestation of tropical and temperate forests throughout their history. Figure adapted from Williams (2002).

area still remain high as ca. 13 million ha of forests were annually removed during the period 2000 - 2010. These losses can be almost completely attributed to deforestation in tropical regions, as the removal of temperate forest area strongly decreased during the twentieth century (Figure 1.1, Williams 2002).

1.1.1 The fragmented forest landscape in Western Europe

Current forest areas in all of Europe are stable or increasing, covering about 32.2% of the land in Europe (without the Russian Federation), nevertheless they bear the imprint of historical human-induced land-use changes (Forest Europe 2011). Especially the densely populated and highly urbanized regions in North-western Europe experienced several periods of strong deforestation, which coincided with changes in economic and social development. The extremely fragmented temperate forests in the northern part of Belgium (Flanders) are representative for this drastic modification of the forest landscape (Vandekerkhove 2013). In Flanders, the amount of forest cover reached a first bottom line during the Roman period, with ca. 12% of the land area forested. After the fall of the Roman Empire in the fifth century, forests recovered during the ensuing centuries. The Flemish forest landscape was further shaped by several cycles of deforestation and afforestation, which resulted in a minimum forest cover of less than 10% around 1300, and a dramatically low amount of forest area (6%) during the industrial revolution (1775 - 1880) (Tack et al. 1993; Hermy & Verheyen 2007). At the end of the nineteenth century, the rate of deforestation decreased, as most of the remaining forests were not suitable for agricultural land and wood was gradually replaced by fossil fuels as primary energy source. Furthermore, and more recently, the reforestation of abandoned agricultural land was encouraged in the context of multifunctional forests by several national and regional policies throughout Europe (Williams 2002).

As a result of the above historical land-use changes, today, Flanders is one of the least forested regions in Europe, with a forest cover of approximately 11% (146,381 ha) (Vandekerkhove 2013). Several periods of deforestation not only reduced the total forest area, but also increased fragmentation of the remaining forests. This has resulted in a complex pattern of forest patches, characterized by

reduced fragment size and limited connectivity between fragments, and which are embedded in a to forest species hostile landscape matrix. The western part of Flanders provides a good example of this historical fragmentation process as the total forest area in this region strongly decreased throughout its history, from more than 20% in 1000 AD to 12.8% in 1775, reaching a minimum forest cover of ca. 2.3% in 1990 (Figure 1.2, Tack et al. 1993). Moreover, the current average forest fragment size in Flanders is small, with 85% of the remnants smaller than 5 ha, and 38% smaller than 1 ha (Van Coillie et al. 2007).

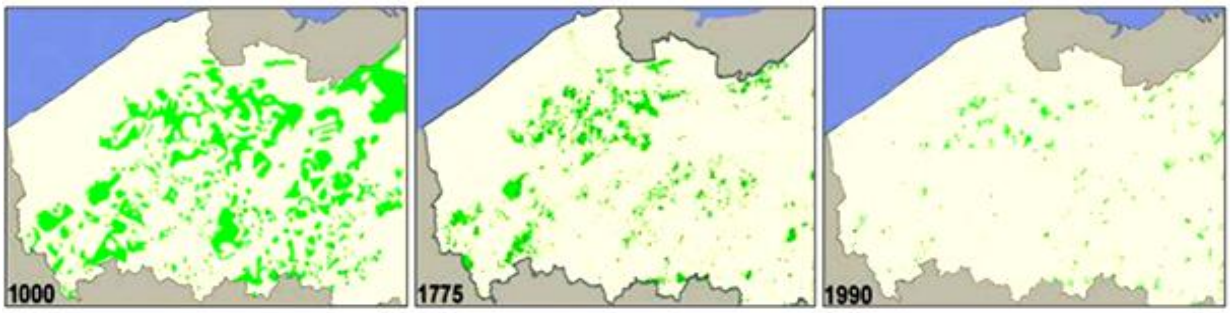

Figure 1.2: Forest fragmentation in the western part of Flanders throughout its history. Figure adapted from Tack et al. (1993).

1.1.2 Forest management

Next to strong deforestation, major socio-economic changes have induced structural shifts in forest management practices (Riitters et al. 2000; Williams 2002). In Europe, forestry evolved from the production of mainly timber during industrialization, to a balanced management of multiple forest goods and services in more recent times. At first, timber was simply harvested from natural forests, and forest management was primarily focused on controlling the rate and pattern of wood exploitation. By the beginning of the industrial revolution, timber stocks were becoming exhausted, after which considerable efforts were made to restore European forests. Forestry shifted from a passive to a more active forest management, in order to ensure a steady supply of timber. During this period artificial reforestation and afforestation resulted in production monocultures, including trees of similar ages, and rotation cycles that maximized wood production (Farrell et al. 2000). In the middle of the twentieth century, the public attitudes towards forests dramatically changed. Forestry shifted towards a more close-to-nature forest management with strong emphasis on the

conservation and sustainable use of forest products (Lindenmayer et al. 1999; Forest Europe 2011). Today, this type of forest management is strongly encouraged in Flanders and many parts of Western Europe, and it has promoted the transformation of production monocultures into a fine-grained forest landscape with more mixed, multifunctional and uneven-aged forest stands (ProSilva 1999; Maes et al. 2011). In general, these shifts in forest management practices may influence the stand structure of forests, and therefore may have profound effects on the tree densities and population sizes of forest stands (Finkeldey & Ziehe 2004).

1.2 Genetic consequences of habitat fragmentation and reduced plant density

Habitat fragmentation is often recognized as the main driving force behind the loss of biodiversity in many terrestrial ecosystems around the world (Sala et al. 2000). Also at the lowest level of biodiversity, *i.e.* genetic diversity, fragmentation of once continuous habitats can have major consequences for plant species (Young et al. 1996). Theoretically, small and spatially isolated plant populations are expected to lose genetic variation due to a combination of several genetic processes, namely (1) increased random genetic drift, (2) inbreeding and (3) reduced gene flow between habitat fragments (Young et al. 1996; Jump & Peñuelas 2006).

1.2.1 Reduced population size

A decreasing population size may immediately create a genetic bottleneck, as only a small sample of the original gene pool will remain after rapid elimination of, especially, rare alleles (Young et al. 1996; Lowe et al. 2005). Other genetic processes will reduce genetic diversity when plant populations remain small over successive generations. Random genetic drift may alter allele frequencies due to a random sampling of alleles from one generation to the next (Figure 1.3). In addition, elevated levels of inbreeding will further reduce genetic diversity over time (Ellstrand & Elam 1993). In plants, inbreeding arises from increased levels of selfing in self-compatible plants, or through mating among closely related individuals (biparental inbreeding) (Barrett & Kohn 1991). Genetic drift and inbreeding eliminate or fix both rare and

more common alleles. This will reduce the allelic richness and heterozygosity within populations, and may ultimately lead to the fixation of recessive deleterious alleles (Lande 1988). To maintain genetic diversity over generations, the 50 - 500 conservation ''rule'' is often cited as a general guidance among taxa (Franklin 1980; Frankham et al. 2013). According to this guideline, the minimum effective population size (N_e), which is the size of an ideal population (random mating, no migration, mutation and selection), has to be higher than 50 to prevent populations against inbreeding and demographic stochasticity. Whereas, The 500 part of the rule, states that populations with an N_e of 500 individuals are able to retain evolutionary potential, based on the balance between mutation (adding genetic variation) and random genetic drift for a quantative trait (genetic losses). However, many factors may influence N_e in natural populations including variance in family size, age structure and sex ratios (Crow & Kimura 1970), which complicate the estimation of N_e. Furthermore, based on the inbreeding depressions that have been reported in laboratory populations, and based on more recent alternative quantitative genetic theory, the 50 - 500 rule should be doubled (100 - 1000 rule) to avoid inbreeding and to limit the total fitness loss (< 10%) in the short term (5 generations) and for maintaining the evolutionary potential of natural populations (Frankham et al. 2014).

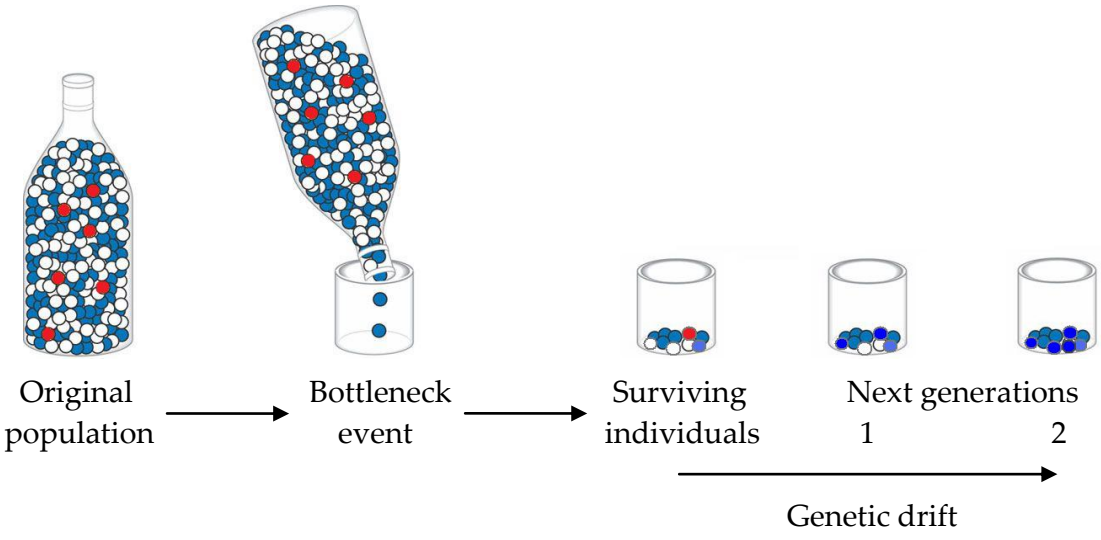

Figure 1.3: Schematic representation of a genetic bottleneck following fragmentation, and the effect of genetic drift on allele frequencies in subsequent generations. Different colours represent different alleles. By chance, blue alleles may become overrepresented (and eventually fixed) in subsequent generations.

1.2.2 Decreased connectivity between habitat fragments

Next to reduced population sizes, increased isolation between patches of suitable habitats may further increase extinction risks, as species distribution patterns are disrupted and dispersing individuals are forced to cross a hostile landscape matrix (Ewers & Didham 2006). Therefore, the maintenance of gene flow through pollen and seed movement plays an important role in conserving genetic diversity in small and isolated plant populations. Genetic connectivity among habitat remnants may increase the effective population size of small populations through the supplementation of gene pools with alleles from neighboring populations (Ellstrand & Elam 1993). This may counteract the negative effects of genetic drift and inbreeding on allele frequencies and on the genetic diversity of small plant populations (Tallmon et al. 2004). To mitigate changes in allele frequencies due to genetic drift, only low levels of gene flow (1 migrant per generation in an idealized population) are required (Wang 2004). However, higher gene flow levels have been suggested to be necessary for preventing genetic differentiation among plant populations due to inbreeding and local adaptation (5 - 20 migrants, Lacy 1987). The amount of gene flow among remnant populations will depend on the degree of geographical isolation between habitat fragments, the quality of the intervening landscape matrix and the diversity of pollen and seed sources that contribute to the local gene pool (Sork & Smouse 2006; Bacles & Jump 2011). Moreover, since gene flow estimates are based on the infinite island model of Wright (1931), violation of model assumptions can produce biased estimates. For example, when the diversity of pollen and seed sources from neighboring fragments that contribute to the local gene pool is restricted, high rates of gene flow will not necessarily protect a population against genetic bottlenecks (Sork & Smouse 2006).

1.2.3 Effect of plant density

Human-mediated changes in population density may also have profound effects on mating and gene flow patterns within populations (Eckert et al. 2010). Reduced local plant density may alter mate availability, such that the number of near neighbours surrounding a focal plant decreases, and the pollination distance between mates

increases. When the distance between neighbouring plants exceeds the scale of pollen dispersal, pollen exchange within the population will be much more restricted, and plants will become reproductively isolated (Sork et al. 2002; Van Rossum et al. 2004). A low diversity of the local pollen pool may increase the relatedness in the next generations, leading to mating among closely related individuals and inbreeding in subsequent generations (Young et al. 1996). Furthermore, in low-density populations of self-compatible plants, or in populations with a pre-existing genetic structure, pollen limitation may directly increase levels of selfing and biparental inbreeding, as proportionally more pollen in the local pollen pool will originate from closely related individuals (Breed et al. 2013b). The extent to which a plant's mating system shifts from outcrossing to selfing (reproductive assurance strategy) will determine the evolutionary outcome of pollen limitation (Eckert et al. 2010). Ultimately, smaller pollen loads may not only have fitness costs through increased selfing and biparental inbreeding, but may also decrease pollen competition, increasing the proportion of recessive deleterious alleles within the local pollen pool (Goubitz et al. 2002).

1.2.4 Relationship between genetic diversity and fitness

Maintaining genetic diversity within populations is of fundamental importance for the performance, persistence and evolution of plants in fragmented landscapes (Jump et al. 2009). The elimination of rare, potentially adaptive alleles, may increase the genetic vulnerability of fragmented plant populations to changing environmental conditions over time (Young et al. 1996; Lowe et al. 2005). Although these low-frequency alleles might not confer a fitness advantage under current environmental conditions, they may be beneficial for plant fitness and survival under changed selection pressures in future populations (Barret & Schluter 2008). Next to the loss of low-frequency alleles, increased levels of consanguineous mating and genetic drift may lead to the fixation and accumulation of deleterious recessive alleles in the homozygous state. This "genetic load" can affect the fecundity, seedling establishment, and survival of individuals, and may further reduce the population size and viability of fragmented tree populations (Husband & Schemske 1996).

Theoretically, the above processes can lead to a genetic extinction vortex as environmental, demographic and genetic factors exacerbate one another in a downward spiral, driving populations to extinction (Ellstrand & Elam 1993; Figure 1.4). However, empirical evidence of genetic extinction vortices in natural populations are scarce, and it has been suggested that other factors (such as environmental stochasticity) may drive a population to extinction before deleterious genetic changes become evident (Matthies et al. 2004).

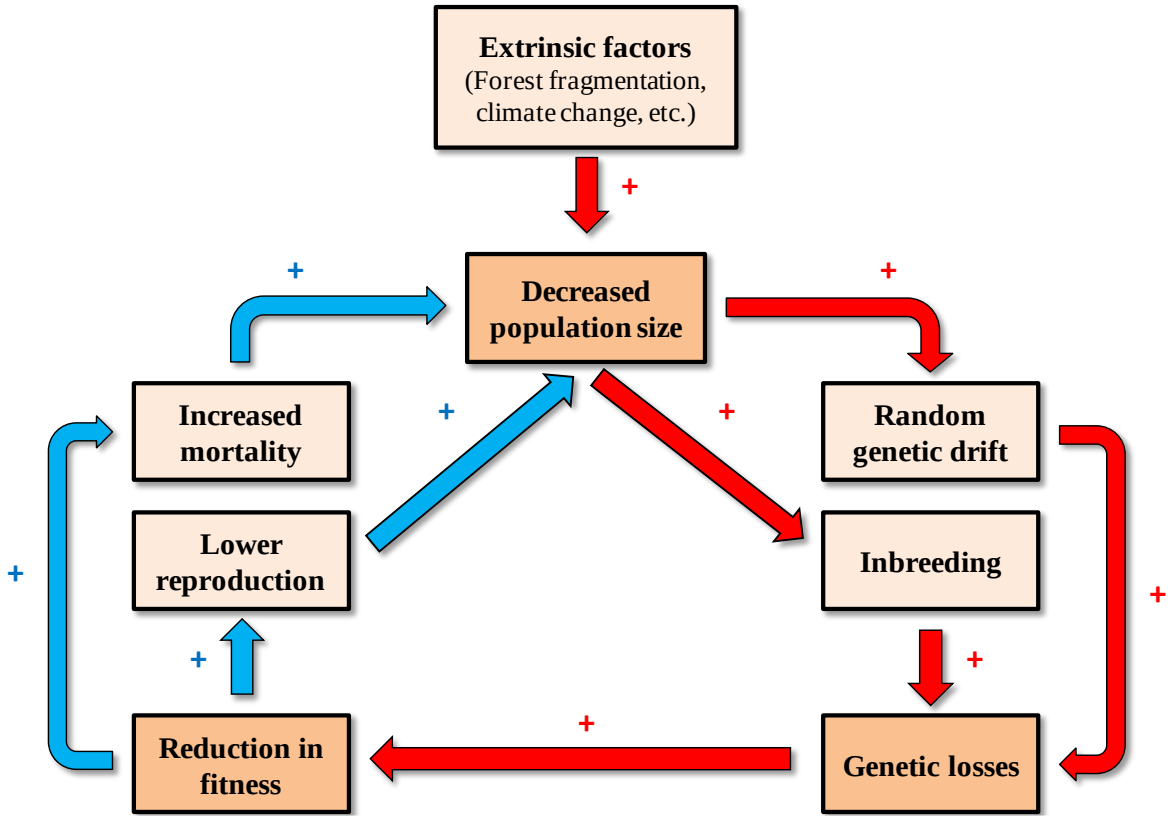

Figure 1.4: The extinction vortex of the small-population paradigm. Theoretically, small populations will fall into a vortex of positive feedback loops in which genetic losses and subsequent reductions in fitness will further reduce population size.

Although one would expect that reductions of individual fitness (i.e., inbreeding depressions) are expressed more readily in self-compatible species, strong selection against inbred individuals may reduce the frequency of deleterious recessive alleles within populations of self-compatible plants (Keller & Waller 2002). Such "purging" of genetic load will be less likely in outcrossing plant species, as deleterious recessive alleles are masked through higher levels of individual

heterozygosity, which will lead to more pronounced inbreeding depressions in fragmented plant populations. In addition, purging will be less efficient to mildly deleterious alleles, which make up most of the genetic load in plants, and will depend on several demographic (population size, gene flow) and genetic factors (Young et al. 1996; Bijlsma et al. 1999).

1.3 Genetic effects of population fragmentation on trees

Tree species are characterized by one of the highest levels of genetic diversity of all plant species. Contrary to herbaceous plants, most of this genetic variation is distributed within populations, while genetic differentiation among populations is on average low (Hamrick et al. 1992). The usual explanation for this general pattern, is that tree species exhibit certain life-history traits, which make them less susceptible to genetic erosion and that promote the maintenance of high levels of within-population diversity (Hamrick 2004). However, the idea that trees are relatively resistant to genetic erosion following forest fragmentation, remains highly debated in forest conservation genetics (Kramer et al. 2008).

1.3.1 Life-history traits

Life-history traits such as high longevity, broad phenotypic plasticity, strong outcrossing and extensive gene flow may influence the genetic response of trees to forest fragmentation (Hamrick 2004). Tree species generally display long generation times and delayed reproduction, through which they may persist with low numbers in forest remnants for a long time, without showing direct genetic losses due to genetic drift and inbreeding. As indicated earlier, the impact of both processes on genetic diversity will depend on the number of generations that a population is subjected to fragmentation conditions. Consequently, short-lived plant species are expected to show stronger negative effects of genetic drift and inbreeding in the short term than long-lived tree species (Young et al. 1996). This idea is, however, complicated by the fact that long-lived plant species may reproduce in overlapping generations, which may increase matings among close relatives in tree species (Finkeldey & Ziehe 2004). Next to long lifespans, trees also display high levels of

phenotypic plasticity, which enable them to respond to a wide variety of ecological conditions on timescales shorter than an individual's lifetime (Jump & Peñuelas 2005). Especially in sessile long-lived organisms, phenotypic plasticity is crucial for survival during periods of adverse and disturbed environmental conditions following forest fragmentation (Hampe & Petit 2005; Valladares et al. 2007).

Perhaps the most important factors that may alleviate the genetic consequences of population fragmentation are high outcrossing rates and extensive gene flow. First, in many tree species outcrossing is enforced by mechanisms such as dioecy and self-incompatibility (Hamrick & Godt 1992). Dioecy and self-incompatibility mechanisms are more frequently present in woody plant species than in herbaceous species, as the reproductive assurance benefit of selfing is of less importance in long-lived plant species (Ehrlén & Lehtilä 2002; Vamosi & Vamosi 2004). In addition, given that adult trees may reproduce during most of their long lifespan and given the large number of offspring that are produced upon which selection may work, strong inbreeding depression, and thus high mortality rates at the seedling stage, may be an acceptable strategy to promote the presence of outcrossed adults in later life stages (Husband & Schemske 1996; Kaufmann et al. 1998). Besides strong outcrossing, woody plant species also have a high potential for gene flow (Hamrick 2004). Due to their large size, trees will not only produce immense amounts of pollen and seeds, but their tall stature will also promote the pollen and seed dispersal process. The higher release height of propagules strongly increase the dispersal distance of wind-dispersed pollen and seeds, while the strong production of flowers and fruits will attract large quantities of animal pollinators and seed dispersers (Nathan et al. 2002; Ghazoul 2005). Especially gene flow via pollen has been suggested to be extensive in both wind- and animal-pollinated tree species (pollen flow over 5 - 10 km), and thus should buffer tree populations against genetic losses following fragmentation (Nason & Hamrick 1997; Hamrick 2004). However, besides this long-distance component, short-distance pollen and seed flow will also play an important role in local recruitment patterns (Chybicki & Burczyk 2010).

1.3.2 The paradox of forest fragmentation genetics

Although numerous studies on woody plant species have supported the common view that trees are relatively resistant to genetic losses following forest fragmentation (Hamrick 2004), this has been questioned by a growing body of research showing negative fragmentation effects on gene flow, inbreeding and genetic diversity (Kramer et al. 2008; Pautasso 2009; Bacles & Jump 2011). This so-called "paradox of forest fragmentation genetics" can be attributed to several confounding factors, which entangle the genetic response of tree species to anthropogenic changes. First, given the long generation time of trees and the relative recent fragmentation of forests (i.e., < 200 years), the adult cohorts of forest fragments often predate habitat fragmentation, which complicate the detection of genetic losses (Young et al. 1996). This implies that recently fragmented tree populations still have to lose a considerable amount of genetic variation in the future, creating a genetic extinction debt (Honnay et al. 2006).

Second, the negative genetic effects of forest fragmentation may differ between temperate and tropical tree species. Temperate forests are less species diverse than tropical forests, through which they exhibit higher densities and population sizes of conspecific trees within forest remnants. As a consequence of this high mate availability, temperate trees should suffer less from the genetic effects of forest fragmentation than tropical trees. However, since tropical tree species were subjected to low tree densities during their evolutionary history, they are assumed to be stronger adapted to long-distance gene flow, and thus better buffered against genetic isolation (White et al. 2002; Hardy et al. 2006).

Finally, tree species can vary widely in their biological attributes. Consequently, a thorough understanding of life history traits, such as pollination and seed dispersal biology, are necessary to understand or predict the genetic response of trees to habitat loss (Montoya et al. 2008; Breed et al. 2013b). For example, animal-pollinated tree species are expected to be more susceptible to habitat fragmentation than wind-pollinated species, as changes in landscape configuration will disrupt unique biotic interactions between trees and their pollinators (Nason & Hamrick 1997; Farwig et al.

2008). A modification of the composition, abundance and behaviour of animal pollen vectors, can ultimately influence pollen flow and decrease genetic connectivity between populations. Nevertheless, also in wind-pollinated tree species mixed empirical signals of fragmentation of tree populations have been found (Bacles & Jump 2011). Although some studies demonstrated evidence of pollen limitation in wind-pollinated species (Sork et al. 2002; Fernández-Manjarrés & Sork 2005; Jump & Penuelas 2006), others found similar or even increased levels of pollen flow within and among forest fragments (Bacles et al. 2005). Reduced population sizes and tree densities can increase the number of long-distance pollination events, which may lead to an enhanced realized gene flow, as a greater proportion of pollen in the local pollen pool will come from distant trees (Robledo-Arnuncio et al. 2004; Wang et al. 2010). Furthermore, the opening up of the landscape may improve air movements within and between forests, and thus enhance pollen flow, counteracting the negative genetic impacts of forest fragmentation (Okubo & Levin 1989; Sork & Smouse 2006). In general, when the scale of isolation between neighbouring and distant trees exceeds the scale of long-distance pollination, pollen limitation may occur, even in wind-pollinated tree species (Sork & Smouse 2006).

1.4 Genetic effects of forest management on trees

The amount and distribution of genetic diversity within tree populations can also be strongly modified as a result of forest management practices (Finkeldey & Ziehe 2004). First, the method of forest regeneration will have strong consequences for the genetic make-up of a tree population. Artificial forest regeneration, through sowing or planting, can be expected to induce stronger genetic changes than natural regeneration, since it fundamentally disrupts the transmission of genes from one generation to the next (Muona & Harju 1989). Moreover, permanent selection on the natural regeneration will favour better adapted local individuals in natural regenerated forest stands (Lefèvre 2004).

Second, in species with very high fecundity and high mortality at early life stages, the variance in reproductive success will be high in natural regenerated forest

stands (Hedrick 2005). Consequently, since variance in family size among seedlings may influence the N_e of a population, we can expect that N_e/N ratios will be much lower in natural regenerated forest stands compared to artificial regenerated seedling cohorts.

Third, regulation of the stand structure through silvicultural treatments can rapidly reduce tree densities of forest stands, and may in turn have profound impacts on mating and gene flow patterns within tree populations (Eckert et al. 2010). For example, under classic shelterwood systems, trees are progressively removed to increase light availability and to promote regeneration ("preparation and establishment cut"). Nevertheless, this gradual removal of adult trees will directly reduce the size and density of the standing population, through which the number of local mating partners within a forest stand decreases and the distance between neighbouring trees increases (Breed et al 2013b). As indicated earlier, this may lead to less diverse local pollen pools, and can ultimately reduce the effective population size and genetic variation of tree populations (Young et al. 1996).

In a context of close-to-nature forest management, the above genetic effects of low conspecific tree density are increasingly expected to occur in future forests, as a result of the transformation of production monocultures into more mixed and multifunctional forest stands (ProSilva 1999; Maes et al. 2011). Moreover, such close-to-nature forest stands will often have an uneven-aged structure, which will increase the opportunity for reproduction in overlapping generations, especially in tree species with limited pollen or seed dispersal (Finkeldey & Ziehe 2004). Consequently, this may enhance mating among relatives and lead to inbreeding and stronger pronounced inbreeding depressions in the offspring cohort.

1.5 Heterozygosity-fitness correlations (HFCs) in trees

Increased homozygosity resulting from inbreeding may affect individual tree fitness, through influencing morphological, physiological and life-history traits. The latter relationships are generally known as heterozygosity-fitness correlations (HFCs), and have been investigated for several decades in a large number of organisms, ranging

from bivalve moluscs to pine trees (Ledig et al. 1983; Bush et al 1987; Zouros 1987; David & Jarne 1997). Empirical evidence has shown that these heterozygosity-fitness correlations (HFCs) are often absent or very weak, only explaining a small proportion (0.07 - 3.3%) of the total variance of a fitness trait (Balloux et al. 2004; Szulkin et al. 2010). The large amount of null results in the literature suggests that some species are more suited to examine HFCs than other (David et al. 1998). Tree species are expected to exhibit stronger relationships between genetic diversity and fitness traits than other organisms, as a consequence of their sessile nature, flexible breeding systems and high genetic load (Klekowski 1988; Petit & Hampe 2006; González Váro et al. 2012). HFCs also vary strongly among studies. For example, Ledig et al. (1983) found a strong and positive relationship between heterozygosity and growth rate in *Pinus rigida*, whereas in *Pinus sylvestris* no evidences of HFCs were found (Savolainen & Hedrick 1995). The inconsistency of HFCs across species and studies can be attributed on the one hand to the different underlying genetic mechanisms of HFCs (direct, local or general effect) and, on the other hand, to methodological differences between studies.

Three primary hypotheses have been proposed to explain the existence of HFCs in natural tree populations (Figure 1.5, Ledig et al. 1983; David 1998; Hansson & Westerberg 2002). The "direct effect" hypothesis suggests that functional overdominance and partial or total dominance at the loci examined results in a fitness advantage of heterozygous individuals. This is especially important when non-neutral markers such as allozymes are used to quantify genetic diversity, since selection on the allelic variants of an enzyme may directly affect the metabolism and physiology of an individual (Mitton 1997). However, in studies using selectively neutral molecular markers such as microsatellites, the correlation between multi-locus heterozygosity and fitness cannot be readily attributed to functional overdominance (Queller et al. 1993; Savolainen & Hedrick 1995). To account for HFCs in selectively neutral marker studies, two alternative hypotheses have been put forward. First, the "local effect" hypothesis is based on non-random associations (linkage disequilibrium) of neutral marker loci with genes directly coding for fitness traits. Significant levels of linkage disequilibrium may arise as a result of genetic

drift, selection or migration. Also demographic processes, such as bottlenecks or founder events followed by rapid population growth may increase linkage disequilibrium in natural populations (Weir & Hill 1980; David 1998). Second, the "general effect" hypothesis suggests that inbreeding will increase homozygosity over the whole genome, allowing the expression of recessive deleterious alleles and associated fitness costs (i.e., classical inbreeding depression) (David 1998; Hansson & Westerberg 2002; Szulkin et al. 2010). Both hypotheses rely on associative overdominance either at the linked fitness loci (local effect) or at genome wide distributed loci (general effect), and are more likely to occur in small nonrandom-mating populations (Szulkin 2010).

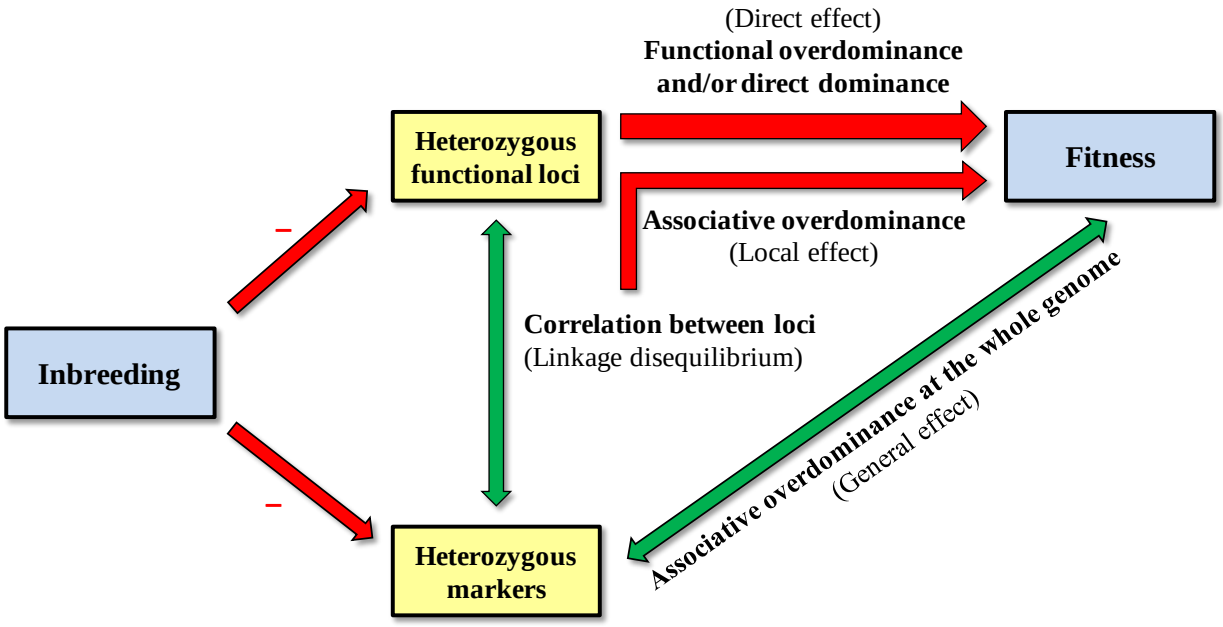

Figure 1.5: Schematic representation of the three primary hypotheses explaining how HFCs arise from inbreeding. Green arrows represent statistical correlations. Figure adapted from Szulkin et al. (2010).

Next to these different hypotheses, the strength of HFCs may also be influenced by several other factors. First of all, the fitness trait under study has been put forward as an important source of variation. Phenotypic traits such as the growth rate and survival of seedlings, which exhibit directional dominance and are typically controlled by numerous loci, are expected to show stronger heterozygosity-fitness correlations than morphological traits (Houle 1998). Second, when phenotypic traits are measured directly in the forest, they may be influenced by several sources of

environmental variation, obscuring the relationship between heterozygosity and fitness. Whereas under a controlled greenhouse environment, variability in fitness traits will better reflect its genetic basis (Ledig et al. 1983, López-Pujol et al. 2008). A final source of variation in HFCs can be attributed to the age of the studied individuals. HFCs will be stronger in early life-cycle stages, as growth and survival are stronger affected in seedlings than in adult trees (David 1998), and because less fit homozygotes, still present in the seedling cohort, may be absent in the adult generation (Honnay et al. 2008).

1.5.1 The effect of environmental stress on HFCs

It is frequently claimed that HFCs are more pronounced in stressful environments than under optimal conditions. Although some studies have demonstrated significant interactions between environmental stress and HFCs (Armbruster & Reed 2005), others have failed to find such an inbreeding-environment interaction, through which this topic remains controversial (Fox et al. 2011). The contrasting findings of empirical studies can be explained by the type and level of stress to which organisms are exposed. Environmental stress can occur under a wide range of conditions (drought, light, competition, etc.), and only above a certain threshold level, the effect of stress on HFCs may become apparent (Fox & Reed 2011). The interaction between environmental stress and HFCs may become particularly important under climate change, which will, for example, increase the number and intensity of drought events in temperate forests (Stocker et al. 2013). During such events, the negative genetic effects of inbreeding on the individual tree fitness may be exacerbated by drought stress, and could ultimately lead to mortality, especially in the seedling cohort (Bond 2000).

1.6 Aims and thesis outline

At present, the effects of forest fragmentation and forest management practices on the genetic diversity of tree populations remain highly debated, resulting in what is known as the "paradox of forest fragmentation genetics" (Kramer et al. 2008). A better understanding of the processes that shape genetic diversity across generations

within tree populations is therefore indispensable for the evaluation and conservation of genetic variation in current and future forests. In addition, examining the relationship between genetic diversity and fitness under different environmental stress conditions, may give insight into how increased inbreeding (reduced heterozygosity) may affect individual tree performance in benign and stressful environments.

Consequently, the general aim of this thesis was to contribute to solving the paradox of forest fragmentation genetics, on the one hand by conducting a systematic and quantitative review of the available literature (meta-analysis), and on the other hand through studying the maintenance of genetic diversity, contemporary gene flow and individual tree performance in small and fragmented pedunculate oak stands in Northern Belgium (Figure 1.6). Currently, studies that have examined and integrated the processes that shape genetic diversity within several fragmented forest stands are relatively rare, especially in temperate, broadleaved tree species. The extremely fragmented temperate forests in the northern part of Belgium (Flanders) provides a good study area for this kind of research.

As study species, we selected the temperate broadleaved tree species pedunculate oak (*Quercus robur* L.), which is a common and economically important tree species of many European forest ecosystems. In Flanders, *Q. robur* is the most predominant broadleaved tree species, with monospecific pedunculate oak stands covering almost 5% of the total forest area (7,173 ha) and with an estimated total growing stock of 3,605,000 m^3 (Jacques & De Cuyper 1998; Waterinckx 2001). Furthermore, pedunculate oak is also a major constituent of mixed forests and plays an important role in the reforestation and afforestation of forest stands, as more than 40% of new forest sites in Flanders are planted with oak trees (Coart et al. 2001). *Q. robur* exhibit a high level of genetic diversity that is amongst the highest of all tree species (Kremer & Petit 1993). This can be attributed to the nearly complete gametophytic self-incompatibility system through which selfing rates are low (2 - 5%; Steinhoff 1993), and to the potential for long-distance pollen flow which is enhanced by the very small (26 - 29 μm, Rushton 1976) and light pollen of pedunculate oak that

is dispersed by wind. Moreover, since large numbers of offspring are produced during the long lifespan of *Q. robur*, selection will be efficient, promoting the presence of outcrossed adults and the maintenance of genetic diversity in later life stages (Husband & Schemske 1996; Kaufmann et al. 1998). In monospecific *Q. robur* stands, however, the recruitment window that contribute to the future adult cohort can be much more restricted, as seedlings require high irradiance and thus forest canopy opening for their further growth and development (Vera et al. 2006; Bary-Lenger & Nebout 1993).

More specifically we aimed to answer the following questions:

- To which extent are woody plant species at risk to lose genetic variation through forest fragmentation?
- Is the loss of genetic variation in woody plants following habitat fragmentation mediated by life-history traits such as mating system, longevity, and seed and pollen dispersal vectors?
- How much genetic diversity is maintained between adults and recent naturally established oak seedlings in fragmented *Quercus robur* stands?
- Is the intergenerational transmission of genetic variation in oak stands mediated by stand characteristics and how is this process affected by patterns of contemporary gene flow?
- What is the role of stand characteristics such as population size, tree density, spatial isolation and the type of the landscape matrix on local pollen pool diversity and mating system parameters of pedunculate oak?
- Is there a relationship between the level of genetic diversity and individual tree performance in oak? Is this relationship stronger under environmental stress conditions?

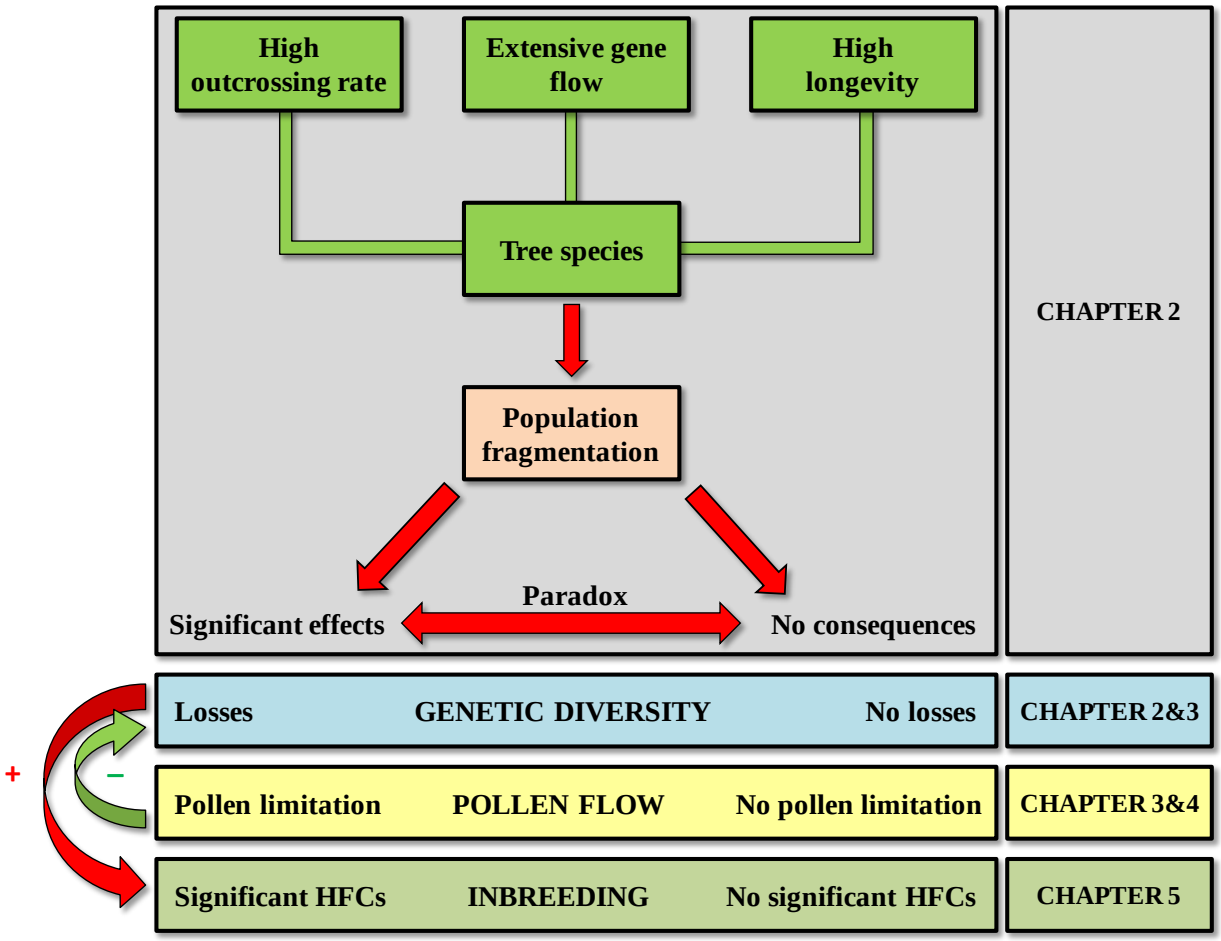

Figure 1.6: Schematic overview of this thesis in which we tried to disentangle the paradox of forest fragmentation, on the one hand by conducting a meta-analysis (**chapter 2**) and on the other hand by studying the effects of fragmentation on genetic diversity, pollen flow and fitness of pedunculate oak stands (**chapter 3,4 & 5**).

The different aims in relation to the different chapters of this thesis are schematically presented in Figure 1.6. Since a systematic and quantitative review of the genetic effects of forest fragmentation on woody plant species was completely lacking in the literature, a meta-analysis was conducted on this topic **(chapter 2)**. In this meta-analysis we examined if the genetic diversity of woody plants was lower in small and fragmented populations compared to large continuous ones. Moreover, we evaluated the influence of several life-history traits (e.g. pollen and seed dispersal vector and longevity) on the genetic response of woody plants to habitat fragmentation. In **chapter 3** the transmission of genetic diversity in pedunculate oak stands in Belgium was investigated through comparing genetic diversity between adults and recently established seedlings in four small oak stands (< 4.5 ha) that

varied in tree density. We were interested whether the benefits of natural regeneration of *Q. robur* were jeopardized in small and low-density forest stands, as the genetic diversity of the standing population could be affected by genetic drift and inbreeding. In this chapter we also quantified mating patterns and gene flow to better understand the processes that shape genetic diversity across generations in the studied oak populations. The latter processes were investigated in more detail in **chapter 4**, where we studied the impact of different stand characteristics (population size, tree density, isolation and type of the landscape matrix) on mating patterns and pollen-mediated gene flow of pedunculate oak. In **chapter 5** we focused on the potentially interacting consequences of habitat fragmentation (low genetic diversity) and climate change-induced drought stress, on the individual fitness of pedunculate oak seedlings. Therefore we performed a greenhouse experiment to investigate the relationship between heterozygosity and fitness traits (transpiration and growth traits) in *Q. robur* seedlings, and examined whether the fitness responses of oak seedlings to low levels of heterozygosity were more pronounced under drought stress than under optimal water conditions. In the final chapter, **chapter 6**, the main findings of the previous chapters were summarized and discussed. Based on this information, we evaluated the current and future genetic risks faced by fragmented pedunculate oak stands and highlighted some forest management practices that could potentially maximize genetic diversity in small fragmented forest stands. Finally, some suggestions for future research were proposed

Chapter 2.

Meta-analysis of susceptibility of woody plants to loss of genetic diversity through habitat fragmentation

Adapted from:

Vranckx G, Jacquemyn H, Muys B, Honnay O. 2012. Meta-analysis of susceptibility of woody plants to loss of genetic diversity through habitat fragmentation. *Conservation Biology* 26: 228-237.

2.1 Abstract

Shrubs and trees are assumed less likely to lose genetic variation in response to habitat fragmentation because they have certain life-history characteristics such as long lifespans and extensive pollen flow. To test this assumption, we conducted a meta-analysis with data on 97 woody plant species derived from 98 studies of habitat fragmentation. We measured the weighted response of four different measures of population-level genetic diversity to habitat fragmentation with Hedge's *d* and Spearman rank correlation. We tested whether the genetic response to habitat fragmentation was mediated by life-history traits (longevity, pollination mode, and seed dispersal vector) and study characteristics (genetic marker and plant material used). For both tests of effect size habitat fragmentation was associated with a substantial decrease in expected heterozygosity, number of alleles, and percentage of polymorphic loci, whereas the population inbreeding coefficient was not associated with these measures. The largest proportion of variation among effect sizes was explained by pollination mechanism and by the age of the tissue (progeny or adult) that was genotyped. Our primary finding was that wind-pollinated trees and shrubs appeared to be as likely to lose genetic variation as insect-pollinated species, indicating that severe habitat fragmentation may lead to pollen limitation and limited gene flow. In comparison with results of previous meta-analyses on mainly herbaceous species, we found trees and shrubs were as likely to have negative genetic responses to habitat fragmentation as herbaceous species. We also found that the genetic variation in the offspring cohort was generally less than that of adult trees, which is evidence of a genetic extinction debt and probably reflects the genetic diversity of the historical, less-fragmented landscape.

2.2 Introduction

In many parts of the world, increasing demands for food and changes in land use have increased the fragmentation of forests (Millennium Ecosystem Assessment 2005). This has resulted in landscapes with forest patches that are scattered in a matrix of agricultural or urban land and thus in small and spatially isolated populations of trees and shrubs. In small and isolated populations, the combination of increased random genetic drift, inbreeding, and reduced gene flow may substantially reduce genetic variation (Aguilar et al. 2008).

The loss of genetic variation may have both short and long-term detrimental effects on population fitness and viability. In the short term, an increased degree of homozygosity may cause the expression of deleterious recessive alleles, which can decrease individual fitness (i.e., inbreeding depression) (Husband & Schemske 1996; Reed & Frankham 2003). In the long term, lowered genetic diversity can affect the potential of a species to adapt to changing environmental conditions (Willi et al. 2006). The genetic response of plants to habitat fragmentation is strongly affected by their life-history traits. Self-incompatible plant species, for example, are more likely to lose genetic diversity through genetic drift than self-compatible species (Honnay & Jacquemyn 2007; Aguilar et al. 2008).

It has been suggested that tree and shrub species are relatively unlikely to lose genetic variation in response to habitat fragmentation. First, due to their long lifespan woody plants may persist in remnant populations for a long time, even without sexual reproduction (Hamrick 2004; Lowe et al. 2005). As a result, in landscapes with a similar fragmentation history, genetic drift will affect populations of trees and shrubs for a smaller number of generations than is the case for species with short generation times (Young et al. 1996). Second, woody plants are characterized by high levels of phenotypic plasticity, which allows them to adapt to environmental change (Hamrick 2004). In this way, woody species are able to respond to adverse changes in the environment on a time scale shorter than an individual's lifetime (Jump & Peñuelas 2005; Valladares et al. 2007), which allows trees and shrubs to survive in small, often disturbed, habitat fragments. Finally, due

to their large size and massive pollen and seed production, woody perennials have a high potential for gene flow. Pollen flow in tree and shrub species, particularly in species in which pollen is dispersed by wind, can be extensive and thus may counteract loss of genetic variation following habitat fragmentation (Nason & Hamrick 1997; Hamrick 2004).

The idea that woody species are relatively resistant to the loss of genetic diversity has been challenged recently by study results showing that there is limited pollen flow between fragmented populations (Knapp et al. 2001; Sork et al. 2002). Habitat fragmentation is associated with increased levels of inbreeding, high population divergence, and reduced genetic diversity in fragmented stands of common beech (*Fagus sylvatica*) (Jump & Peñuelas 2006). Similar results have been reported for wind-pollinated oak species (*Quercus douglasii*, *Q. lobata*, & *Q. robur*), where forest fragmentation has reduced the number of available pollen donors and limited pollen dispersal (Knapp et al. 2001; Sork et al. 2002; Vakkari et al. 2006).

However, no systematic and quantitative review of the effects of habitat fragmentation on the genetic diversity of woody plant species has been conducted. We performed a meta-analysis of 98 studies that reported data on the relation between population size and fragmentation status or population-level genetic diversity. We examined the independence of the studies by testing whether a phylogenetic signal and a publication bias were present. Specifically, we examined the following questions:

- Is the genetic diversity of woody plants in small and fragmented populations low relative to large continuous populations?
- Are the genetic effects of habitat fragmentation larger in progeny than in adults?
- Does the genetic response differ among pollination modes (wind, birds, and insects)?
- Is the probability of reduced genetic variation in woody plants following habitat fragmentation associated with other life-history traits such as mating system, longevity, and seed dispersal vectors?

2.3 Materials and Methods

2.3.1 Study selection and coding

In December 2009, we conducted an extensive search of the literature in the Thomson Reuters Web of Science (ISI) database. We used a combination of the following keywords: *habitat fragmentation**, *genetic**, *woody plants**, *trees**, and *shrubs**. We include in our meta-analysis only papers that reported genetic diversity measures, population sizes or fragmentation status, and the number of samples used in the genetic analyses. In the selected studies, genetic diversity was determined with both dominant (amplification fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD)) and codominant (simple sequence repeats (SSR) and allozymes) markers. For codominant data most studies reported the number of alleles per locus (A), percentage of polymorphic loci (P), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}). Because dominant markers do not allow distinguishing a homozygous genotype for the dominant allele from a heterozygote genotype (Mueller & Wolfenbarger 1999), most researchers applying dominant markers reported only P and H_e. When genetic diversity measures were not reported, we extracted raw data from graphs and tabulated it with GetData Graph Digitizer 2.24 (Fedorov 2008). In some cases, F_{IS} was calculated as the ratio between observed and expected heterozygosity.

2.3.2 Univariate data analyses

We used two methods to calculate effect sizes, such that we could include as many studies as possible. For studies reporting population sizes, we calculated the Spearman rank correlation coefficient (r_s) between population size and 4 measures of population-level genetic diversity (A, P, H_e, and F_{IS}). We did not perform a Fisher z transformation on the individual correlation coefficients because this leads to more biased effect sizes (Field 2001). For studies that distinguished clearly between small fragmented populations and large continuous ones, we used Hedge's d as a measure of effect size. We calculated Hedge's d as the unbiased standardized mean difference of genetic diversity metrics between fragmented and continuous habitats as follows:

$$d = \frac{\left(\bar{X}_F - \bar{X}_C\right)}{S} J ,$$

where $\bar{X}_F$ and $\bar{X}_C$ are the mean values of a given genetic parameter in fragmented and continuous habitats, S is the pooled standard deviation of both groups, and J is a factor that accounts for the effects of small sample sizes (Gurevitch & Hedges 2001). A positive Spearman correlation effect size and a negative Hedge's d both imply negative effects of habitat fragmentation on population level genetic diversity. Each study was weighted $((A-2)N)^{1/2}$ according to (Reed & Frankham 2003), where A is the number of populations studied, and N is the mean number of individuals genotyped. We examined the possibility of publication bias graphically. We plotted effect sizes versus study weight in funnel plots and tested the significance of this relation with a Spearman rank correlation test (Light & Pillemer 1984; Palmer 1999).

We used MetaWin 2.1 software (Rosenberg et al. 2000) for the meta-analysis and applied a random effects model. We assessed the heterogeneity among effect sizes with Q statistics (Hedges & Olkin 1985). In particular, we examined the p values of the Q between statistics because we were interested in the variation in effect sizes due to differences between the categories of a moderator variable (Lipsey & Wilson 2001). In addition to Q heterogeneity statistics, we calculated 95% bias-corrected bootstrap CIs (4999 iterations) of the effect sizes across studies (Adams et al. 1997).

2.3.3 Moderating variables

If the Q statistic was significant, we evaluated the significance of the following life-history traits as a moderator variable: dominant pollen vector (wind, insects, or birds); dominant seed dispersal vector (gravity, wind, birds, ants, or a combination of birds and mammals); mating system (obligate outcrossing, self-compatible, but mainly outcrossing or self-compatible); and longevity of individuals of the species (< 100 or > 100 years).

Because the methods we used to assess the effect of habitat fragmentation on genetic diversity may also affect the results, we included sampling method as a moderating variable. We tested whether the marker system used (allozymes, SSRs,

RAPDs, or AFLPs) was associated with the genetic response to habitat fragmentation. Because the effect of habitat fragmentation may depend on the plant material used (stronger genetic responses to habitat fragmentation can be expected when progeny tissue is used), we examined whether genetic diversity of the adult generation differed significantly from that of the progeny. However, plant tissue from adult trees collected in old fragmented forests may have reduced genetic variation; thus, we also incorporated the amount of time elapsed after a fragmentation event in the meta-analysis. Because most authors only gave rough estimates of when fragmentation started, we assigned studies to two categories: > 1 generation or < 1 generation after fragmentation. To test for potential interactions among moderator variables, we measured the pairwise degree of association with chi-square tests (Honnay & Jacquemyn 2007).

2.3.4 Phylogenetic signal

A potential problem when analyzing data on many taxa is their shared phylogenetic history (Gitzendanner & Soltis 2000). A phylogenetic signal may be present because taxa that are closely related tend to be more similar in morphology and ecology than distantly related taxa. Therefore, we also tested for the presence of a phylogenetic signal with phylometa 1.0 beta (Lajeunesse 2009). We calculated phylogenetically independent statistics of fixed and random-effects models and conducted homogeneity tests. We constructed a phylogenetic tree of the studied species in Mesquite 2.72 (Maddison & Maddison 2009) (Appendix 2.1). We used the plant phylogeny of Soltis et al. (2000) as a reference tree. Inter- and infrafamilial and infragenus phylogenetic relations were obtained from Chen et al. (1999), Mast and Givnish (2002), Li et al. (2004), Wojciechowski et al. (2004), Gernandt et al. (2005), Schönenberger et al. (2005), Wilson et al. (2005), Crayn et al. (2006), Weston and Barker (2006), and Potter et al. (2007). For the genera *Acacia*, *Eucalyptus,* and *Grevillea*, we could not resolve infragenus relations, so we left these taxa as soft polytomies. Because it was not possible to obtain dated estimates of divergence times, we applied four arbitrary branch-length assumptions: all branch lengths equal 1; branch length equals the number of species in a clade minus one with all the species constrained to

be contemporaneous (Grafen 1989); species are contemporaneous, but all internode branch lengths are equal to one (Pagel 1992); and branch length from the tip to a current node equals the logarithm (base 10) of the number of tip species descending from that node (Nee's method, Purvis 1995). To allow comparisons, we calculated log-likelihood values and Akaike's information criterion (AIC) for all models. The probability that a given model was the best fit compared with all other models was determined by its Akaike weight (Burnham & Anderson 2001), which is the likelihood of the model divided by the sum of the likelihoods of all models.

2.3.5 Multivariate linear mixed model

We ran a full linear mixed model that included moderator variables and their interactions as fixed effects and the random effect "author", which removed the variability in our data caused by studies associated with the same lead authors. Both Spearman rank correlation and Hedge's *d* effect sizes were fitted with this model for every genetic parameter (A, P, H_e, and F_{IS}). Although this multivariate linear mixed model accounted for the covariance between explanatory variables, this approach also has some major shortcomings. First, the sampling variances in the individual studies are not addressed by weighting. Second, a multivariate analysis would require that for each study we have information on all tested moderator variables, which would reduce the size of the data set considerably. An average loss of data points of 35% and the absence of a study weight reduced power of the multivariate analysis and led to nonsignificant results compared with the results of the univariate analyses. Thus, the results of the mixed models are not reported because their statistical power was limited and no new information was provided.

2.4 Results

2.4.1 Generalities of meta-analysis

We retrieved 98 published studies on 97 woody plants, which yielded 105 data points (Appendix 2.2). Each data point included one or more measures of genetic diversity (Appendix 2.3). Population sizes were reported for 91 data points, whereas only 60 of

the 105 records allowed calculation of Hedge's *d*. There was no evidence of publication bias; funnel plots of effect size versus study weight for the four tested measures of genetic diversity were funnel shaped and symmetrical for both measures of effect size (Appendix 2.4 & 2.5). Moreover, none of the corresponding Spearman rank correlation tests between study weight and effect size, calculated with both Spearman rank correlation (*A*, 0.001; *P*, 0.079; H_e, 0.043; F_{IS}, -0.198) and for Hedge's *d* (*A*, 0.142; *P*, 0.118; H_e, 0.061; F_{IS}, 0.058), were significant ($p > 0.05$ in all cases).

For both measures of effect size, the best model fit (lowest AIC values and highest Akaike weights) was obtained when phylogenetic information was excluded from the meta-analysis (Table 2.1). The four other evolutionary models (Grafen, Pagel, Nee, and branch lengths equal to 1) resulted in similar weighted effect sizes, none of which were significantly different from those of the basic meta-analysis (results not shown). Therefore, we used a standard random-effects model in all subsequent analyses (Rosenberg et al. 2000).

2.4.2 Genetic consequences of habitat fragmentation

The use of different genetic markers to study genetic variation did not significantly influence effect sizes (results not shown). Overall, the mean weighted effect sizes for *A*, *P*, and H_e were significantly different from zero ($p < 0.05$). Spearman rank correlation effect sizes were all positive, indicating that forest stands with small populations had lower genetic diversity than larger populations. Similarly, values of Hedge's *d* were all negative, indicating that genetic diversity in forest fragments was lower than in large continuous fragments (Figure 2.1). The inbreeding coefficient (F_{IS}), however, was not significantly ($p > 0.05$) associated with habitat fragmentation (Figure 2.1).

The following pairwise associations between moderator variables were significant ($p < 0.05$): pollination mode and longevity, pollination mode and dispersal mode, marker and progeny material, and longevity and dispersal mode. However, longevity, seed dispersal, mating system, and genetic marker were not significant effects, which excludes the possibility of misinterpretation of the mediating factors associated with loss of genetic diversity.

Table 2.1. Results of Akaike information criterion (AIC) analyses of 5 evolutionary random-effects models of plant phylogeny (meta-analysis without and with phylogenetic signal (branch lengths = 1, Grafen's, Pagel's and Nee's)).

Measure of genetic diversity and model[a]	Spearman rank correlation[b]			Hedge's d[b]		
	$-2log(L_i)$	*AIC*	w_i	$-2log(L_i)$	*AIC*	w_i
No. of alleles/locus (*A*)						
no phylogeny	196	198	> 0.999	141	143	> 0.999
BL = 1	291	293	< 0.001	209	211	< 0.001
Grafen's	409	411	< 0.001	288	290	< 0.001
Pagel's	314	316	< 0.001	221	223	< 0.001
Nee's	257	259	< 0.001	184	186	< 0.001
Percentage of polymorphic loci (*P*)						
no phylogeny	172	174	> 0.999	87	89	> 0.999
BL = 1	252	254	< 0.001	121	123	< 0.001
Grafen's	357	359	< 0.001	160	162	< 0.001
Pagel's	271	273	< 0.001	126	128	< 0.001
Nee's	220	222	< 0.001	107	109	< 0.001
Expected heterozygosity (H_e)						
no phylogeny	252	254	> 0.999	175	177	> 0.999
BL = 1	382	384	< 0.001	253	255	< 0.001
Grafen's	545	547	< 0.001	347	349	< 0.001
Pagel's	412	414	< 0.001	268	270	< 0.001
Nee's	336	338	< 0.001	226	228	< 0.001
Inbreeding coefficient (F_{IS})						
no phylogeny	201	203	> 0.999	144	146	> 0.999
BL = 1	301	303	< 0.001	216	218	< 0.001
Grafen's	425	427	< 0.001	301	303	< 0.001
Pagel's	325	327	< 0.001	232	234	< 0.001
Nee's	267	269	< 0.001	190	192	< 0.001

[a]*The best model fit (lowest AIC values and highest Akaike weights) was the no-phylogeny model for every genetic parameter.*

[b]*Abbreviations: log(*L_i*), natural logarithm of the maximum likelihood for model* i; w_i, *Akaike weight.*

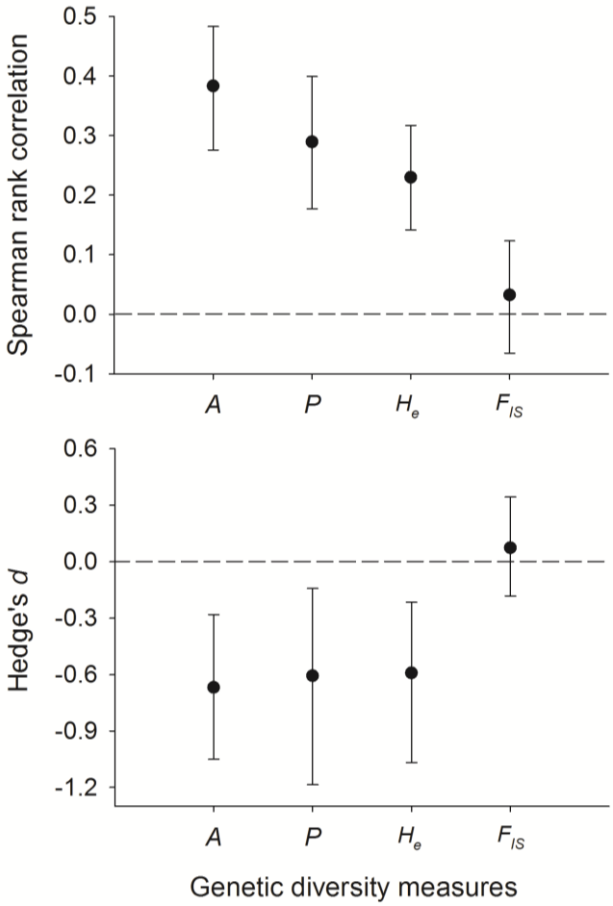

Figure 2.1. Overall weighted mean effect sizes (measured with Spearman rank correlation coefficient and Hedge's *d*) and 95% bias-corrected bootstrap CIs for four measures of genetic diversity in woody plants (*A*, number of alleles per locus; *P*, percentage of polymorphic loci; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient).

For both measures of effect size, the highest proportion of variation in the data set was explained by the pollination vector. The loss of genetic diversity of trees pollinated by wind and insects was more strongly associated with habitat fragmentation than genetic variation of species pollinated by birds. For the correlational measure of effect size, *A* ($Q_{between}$ = 6.11; $p < 0.05$) differed significantly among species pollinated by insects, wind, and birds. Hedge's *d* showed significant heterogeneity for *A* ($Q_{between}$ = 16.66; $p < 0.001$) and H_e ($Q_{between}$ = 6.47; $p < 0.05$) (Figure 2.2).

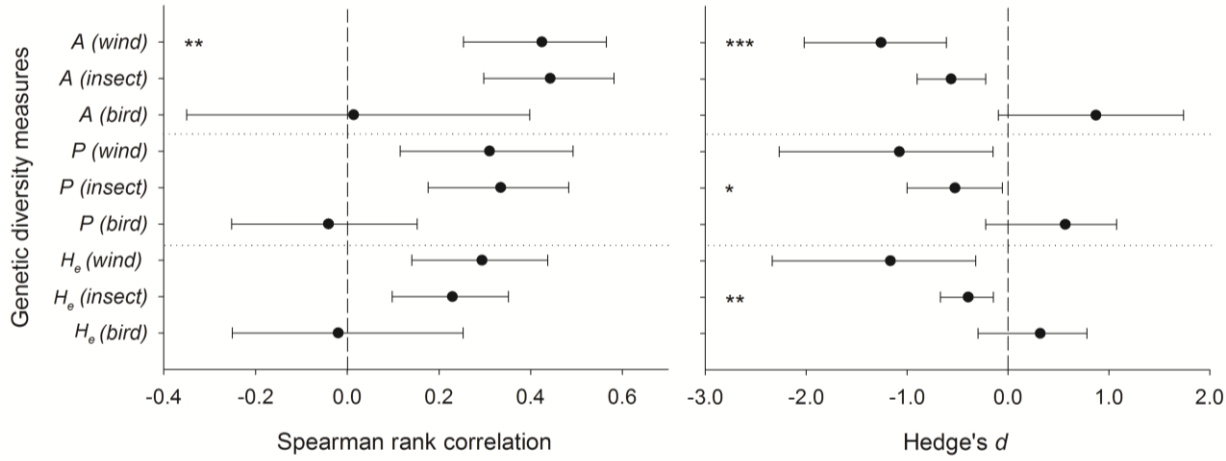

Figure 2.2. Weighted mean effect sizes and 95% bias-corrected bootstrap CIs calculated for different pollination mechanisms (wind, insects, birds) in woody plants (*Q* statistic significance between pollinator groups, * $0.05 \leq p < 0.1$; ** $0.001 \leq p < 0.05$; *** $p < 0.001$; *A*, number of alleles per locus; *P*, percentage of polymorphic loci; H_e, expected heterozygosity).

For the methodological moderator variables, type of plant tissue genotyped (adult versus progeny) differed significantly. For the correlational measure of effect size, *A* for adults was significantly greater than *A* for progeny ($Q_{between} = 6.12$; $p < 0.05$), whereas Hedge's *d* had significantly greater *P* ($Q_{between} = 6.54$; $p < 0.05$) relative to progeny (Figure 2.3). This difference increased when we included the amount of time elapsed after a fragmentation event. Studies in which progeny or adult material was collected in historical habitat fragments in which time of fragmentation exceeded the lifespan of the species had effect sizes that were significantly different from zero for both the Spearman rank correlation and Hedge's *d*. In contrast, adult trees in areas fragmented for < 1 generation did not show signs of loss of genetic variation. The $Q_{between}$ statistics ($p < 0.5$) were significant for the Spearman rank correlation for *A*, *P*, and H_e, whereas for Hedge's *d* the lowest *p* values for $Q_{between}$ ranged between 0.05 and 0.1 for *P* and H_e (Figure 2.4).

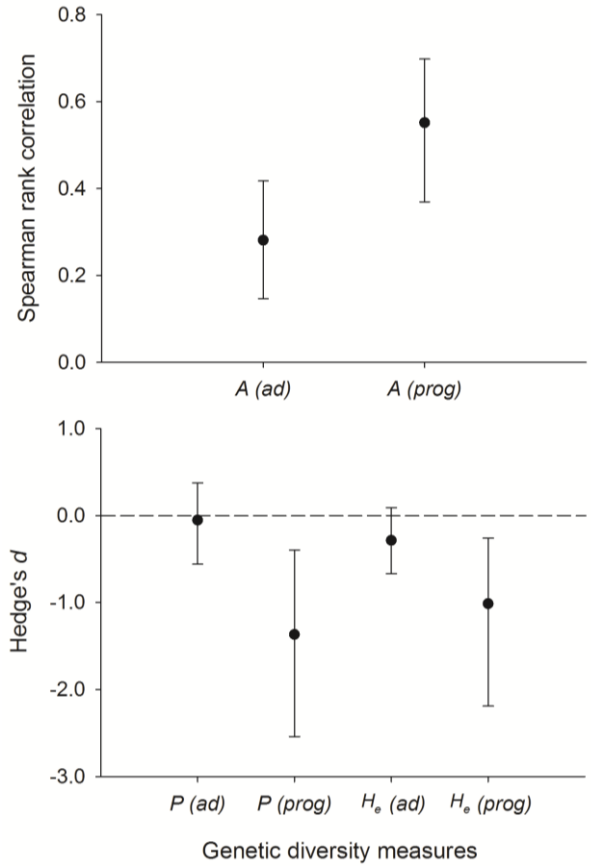

Figure 2.3. Weighted mean effect sizes and 95% bias-corrected bootstrap CIs calculated for adult (ad) and progeny (prog) woody plants. Only genetic parameters with a significant *Q* statistic between groups ($p < 0.05$) (*A* and *P*) and H_e ($0.05 \leq p < 0.1$) are shown for both types of effect size (*A*, number of alleles per locus; *P*, percentage of polymorphic loci; H_e, expected heterozygosity).

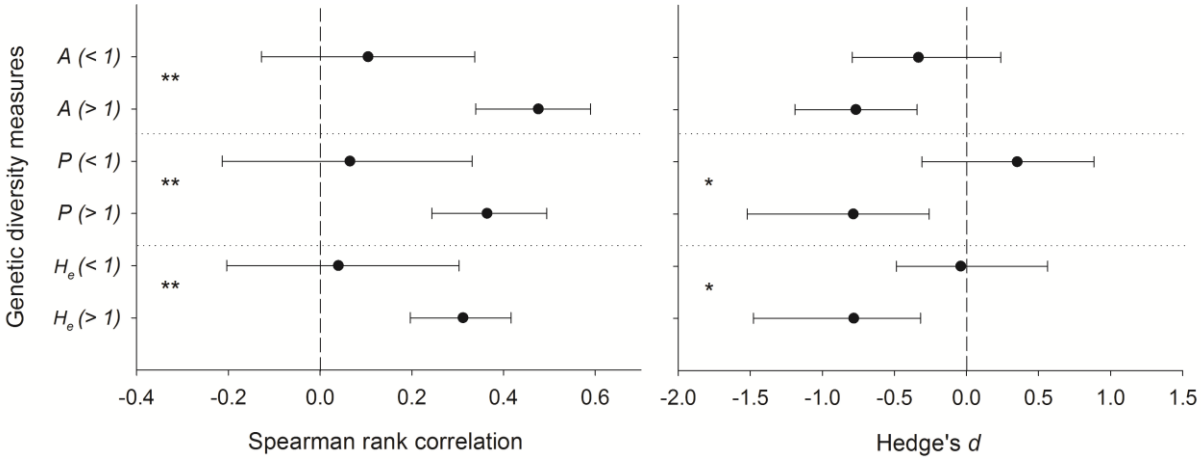

Figure 2.4. Weighted mean effect sizes and 95% bias-corrected bootstrap CIs calculated for habitat fragmentation studies with different amounts of time since fragmentation (< 1 generation versus > 1 generation; significant *Q* statistics between groups, * $0.05 \leq p < 0.1$; ** $0.001 \leq p < 0.05$; *** $p < 0.001$; *A*, number of alleles per locus; *P*, percentage of polymorphic loci; H_e, expected heterozygosity).

2.5 Discussion

Results of our meta-analysis showed that population-level genetic diversity of woody species is negatively affected by habitat fragmentation. Moreover, our results are largely consistent with results of previous meta-analyses conducted by Honnay and Jacquemyn (2007) and Aguilar et al. (2008). This consistency is striking because these authors studied effects of fragmentation on predominantly herbaceous species. The correlation effect sizes of Honnay and Jacquemyn (2007) for *A* (0.36), *P* (0.35), H_e (0.23), and F_{IS} (-0.04) are almost identical to the effects sizes we found. This was also the case for Hedge's *d* values for *A* and *P* (effect size ≈ -0.7) reported by Aguilar et al. (2008). Moreover, our finding that genetic variation of woody plant species was responsive to habitat fragmentation as genetic variation of herbaceous species is consistent with results of recent empirical studies (Knapp et al. 2001; Sork et al. 2002; Jump & Peñuelas 2006) that also challenge the common idea that woody species are relatively unlikely to lose genetic diversity.

The metrics *A* and *P* were most strongly associated with habitat fragmentation; they were more affected to the loss of rare alleles than H_e. Following fragmentation, a reduction in population size will lead directly to a smaller allele pool through the elimination of especially rare alleles (Young et al. 1996; Lowe et al. 2005). Other processes acting over more generations, including random genetic drift and elevated levels of inbreeding, will further reduce genetic diversity (*A*, *P*, and H_e) because both rare and more common alleles are eliminated (Lande 1988; Young et al. 1996).

The inbreeding coefficient (F_{IS}) was not significantly associated with habitat fragmentation. The presence of a Wahlund effect or selection against homozygotes during recruitment could have affected the response of F_{IS}. With a Wahlund effect, heterozygosity is lower than expected as a result of the subdivision of a small, isolated population (Lowe et al. 2004), whereas selection against homozygotes at early life stages could give the impression of heterozygote superiority and corresponding lower inbreeding coefficients (Remington & O'Malley 2000; Hufford & Hamrick 2003). The likelihood of population substructuring will be greater in large populations and lead to upward-biased inbreeding coefficients (Young et al. 1999;

Vakkari et al. 2006). Small and fragmented populations will instead exhibit a heterozygosity excess and consequently a downward-biased F_{IS} value (Raijmann et al. 1994). Eventually the combination of both processes will decrease the differences between the inbreeding coefficients of large and small populations.

2.5.1 Pollination mechanism

Fragmentation was negatively associated with genetic variation in insect-pollinated trees and shrubs. This is consistent with the results of several studies that demonstrate insect pollinators stay within a habitat fragment, rather than moving among fragments (e.g., Didham et al. 1996; Goverde et al. 2002). Moreover, habitat fragmentation has a strong influence on animal pollinators because it affects their population size and foraging behavior (Ashworth et al. 2004), which, in turn, may contribute to the strong association of genetic variation in insect-pollinated trees with habitat fragmentation.

Bird-pollinated species, however, maintained genetic diversity; the mean weighted effect sizes of bird pollination were not significantly different from zero. Movement of pollen by birds among forest fragments may promote outcrossing and provide access to a larger mating pool (Murcia 1996). Kramer et al. (2011) recently reported lower genetic differentiation in Bridges penstemon (*Penstemon rostriflorus*), which is pollinated by hummingbirds, than in Scabland penstemon (*P. deustus*), which is pollinated by bees. This difference indicates birds may more effectively connect distant populations through long-distance pollination. The higher mobility of birds relative to insects might be explained by their higher energy needs. As the number of food sources decreases, birds will have to visit several food sources to satisfy their needs and therefore promote gene flow among sites (Stiles 1978). There is empirical evidence that the foraging of birds among isolated populations is increased by habitat fragmentation because food resources (seeds, nectar) in small remnants are quickly depleted (Bacles et al. 2004; Farwig et al. 2006). In contrast, some argue that pollen flow through birds tends to be very localized (Coates & Hamley 1999; Llorens et al. 2004) because birds visit more flowers within rather than among plants, even in continuous forests. Wind-pollinated trees and shrubs appear

to be as likely to lose genetic diversity as insect-pollinated species, indicating that severe habitat fragmentation may lead to pollen limitation and limited gene flow, even in tree species that are presumed to have efficient ways of gene dispersal. It is likely that due to leptokurtic dispersal of wind-borne pollen, the efficiency of wind pollination will drop sharply as distances from a pollen source increase (Levin & Kerster 1974; Honig et al. 1992). Once a threshold value of spatial isolation has been reached, less pollen will be transferred successfully between forest stands. Moreover, wind pollination within stands may be affected by small population sizes and low habitat quality in fragments. Knapp et al. (2001), for example, observed a decline in acorn production as stand density of blue oak (*Q. douglasii*) decreased. A smaller pollen cloud combined with increasing distances among individuals also decreases the efficiency of pollen transfer within stands (Whitehead 1983; Knapp et al. 2001).

2.5.2 Future loss of genetic variation

The genetic effects of habitat fragmentation were less pronounced in studies in which adult plant tissue was used than in studies in which progeny tissue was used (Figure 2.3). Moreover, studies in which adult plant tissue from populations fragmented for < 1 generation were used showed no significant genetic responses (Figure 2.4), which suggests that the reported genetic diversity reflects that of the historical, less-fragmented landscape and therefore can be considered to be in a prefragmented state. This time lag between forest fragmentation and loss of genetic variation may have created a so-called genetic extinction debt (Honnay et al. 2006). Because woody plants have long life-spans, decreases in their genetic variation are more likely to be affected by these time lags (Vellend et al. 2006; Kuussaari et al. 2009). This implies that recently fragmented tree populations may lose a large amount of their genetic variation, which could eventually lead to local extinction as they pay off their extinction debt. In addition, studies that investigated adult genetic diversity in recently fragmented forest patches, may have seriously underestimated the genetic effects of habitat fragmentation. We found that the response of tree populations to habitat fragmentation depended on the characteristics of the fragmented system. Genetic loss between generations may be limited if populations remain large and

continuous enough so as not to exceed fragmentation thresholds below which genetic variation is lost over time (Ezard & Travis 2006).

The difference between the genetic variation of adults and progeny depended heavily on the genetic diversity measure that was used. For adult and progeny tissue, *A* was significantly associated with habitat fragmentation, whereas for *P* and H_e, the mean weighted effect sizes of adult tissue did not differ significantly from zero. This can be explained by the fact that *A* is more sensitive to the early effects of fragmentation, whereas heterozygosity requires longer periods of fragmentation before its effects become detectable (Young et al. 1996; Kramer et al. 2008).

Chapter 3.

Transmission of genetic variation from the adult generation to naturally established seedling cohorts in small forest stands of pedunculate oak

Adapted from:

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Kint V, Muys B, Honnay O. 2014. Transmission of genetic variation from the adult generation to naturally established seedling cohorts in small forest stands of pedunculate oak (*Quercus robur* L.). *Forest Ecology and Management* 312: 19-27.

3.1 Abstract

Natural regeneration is being increasingly encouraged as the preferred regeneration method for sustainable forestry. However, the benefits of natural regeneration may be jeopardized in small and low-density forest stands, as genetic drift and inbreeding may reduce genetic diversity of the standing population. Particularly in light-demanding tree species, which are characterized by a narrow recruitment window, conservation of genetic diversity during natural regeneration can be challenging in small forest stands. In this study, we investigated intergenerational transmission of genetic diversity in stands of the light-demanding tree species pedunculate oak (*Quercus robur* L.) by comparing genetic diversity between adults and recently established seedlings (1-3 years old) in four small stands of *Q. robur* (< 4.5 ha) that varied in tree density. We also quantified mating patterns and gene flow to investigate their role in shaping the genetic diversity and spatial genetic structure of the populations. When all stands were pooled, the adult trees showed a slightly but significantly higher allelic richness (A_r) than the offspring cohort (A_r = 11.5 and 10.7, respectively). However, at the stand level, no significant negative effects on the genetic diversity of the seedling cohort were found. As expected, acorn dispersal was restricted to a few meters from mother trees, resulting in significant small-scale spatial genetic structure in the progeny. Pollen inflow from outside the study plots varied strongly among stands, but all plots showed a significant correlated paternity (r_p), with higher estimates of r_p found in small and low-density stands. Given that high r_p-levels may increase the probability of biparental inbreeding and may incur fitness costs in subsequent generations, we recommend that in small scale forestry of *Q. robur*, population sizes and tree densities should be sufficiently large to maintain genetic diversity over the long term. Allowing gene flow between tree populations through reducing the spatial isolation among stands, can help to increase N_e in small-scale silvicultural systems of pedunculate oak, but also in other species characterized by a narrow recruitment window.

3.2 Introduction

One of the major challenges in sustainable forestry is the conservation of biological diversity, including forest genetic resources (Lindenmayer et al. 1999). By providing the raw material for evolution, genetic diversity is generally considered important for adaptation of forest trees to changing environmental conditions, and thus for the maintenance of vital and productive forests (Jump et al. 2009). In the face of ongoing climate change, maintaining high genetic diversity within forest stands will increase the likelihood that well adapted genotypes are present and increase the long-term viability of tree populations (Hamrick 2004).

The sessile nature, longevity and high fecundity of tree species imply that the most important changes in the amount and distribution of genetic diversity within forest tree populations can be expected during the regeneration phase. This is because in naturally regenerated forest stands mortality rates of seedlings can be very high (Kaufmann et al. 1998; Vranckx et al. 2012), particularly in light-demanding tree species (Götmark 2007). Although light-demanding seedlings can tolerate low amounts of daylight during the first years of regeneration through surviving on the nutrients in their seeds and storage organs, insufficient canopy opening may rapidly result in massive dying off of seedlings within 3 - 5 years after germination (Lemée 1987; Vera et al. 2006). As a result of this typically small window of recruitment opportunity, selection against less adapted genotypes can be expected and therefore natural regeneration is generally considered as an effective way to maintain genetic diversity and the evolutionary potential of trees (Finkeldey & Ziehe 2004). Compared to artificial regeneration, natural regeneration also has considerable economic and ecological advantages (Bürgi & Schuler 2003; Shono et al. 2007; Chazdon 2008), and the risk for outbreeding depression, which may occur through the transfer of seeds or seedlings from different provenance regions, will be avoided in natural regenerated forest stands (Kramer & Havens 2009).

Natural regeneration is, however, not necessarily a safeguard against losses of genetic diversity between generations because the exact way alleles are transferred from the parental generation to progeny also depends on silvicultural practices

(Robledo-Arnuncio et al. 2004; Piotti et al. 2011), and stand characteristics such as stand size and tree density (Young et al. 1996; Eckert et al. 2010). For example, under classic shelterwood systems, trees are progressively removed to increase light availability and to promote natural regeneration. However, at the same time the effective population size of the parental population is reduced, which may decrease the genetic diversity of recruits on the short term through random genetic drift (Finkeldey & Ziehe 2004). Moreover, removal of adult trees and decreasing tree density may directly affect pollen and seed dispersal patterns. This can, in turn, increase the relatedness in the next generations and result in mating among closely related individuals and increased inbreeding in later generations (El-Kassaby et al. 2003; Sork et al. 2002). The negative effects of reduced stand density on mating patterns were recently supported by a meta-analysis conducted by Breed et al. (2013b). In this study, animal-pollinated woody plants showed less diverse pollen pools and increased selfing rates in forest stands with low tree density. These interactions between tree density and mating patterns are not only found in animal pollinated woody plants, even in wind-pollinated tree species, which are generally characterized by extensive gene flow, positive relationships were found between the density of a forest stand and the estimated number of pollen donors contributing to the offspring (Sork et al. 2002; Fernández-Manjarrés & Sork 2005). Besides changes in within-stand genetic variation and mating system, altered patterns of seed and pollen dispersal associated with changes in stand characteristics can also be expected to affect the spatial genetic structure of tree populations (Vekemans & Hardy 2004). While it has been suggested that distant pollen movement can be facilitated by opening up the landscape (Bacles et al. 2005), small and low-density forest stands, which are generally characterized by large inter-tree distances, are likely to be particularly susceptible to generate a significant spatial genetic structure as a result of limited gene dispersal over greater distances (Hamrick et al. 1993; Sagnard et al. 2011).

Although natural regeneration is likely the best strategy to maintain genetic diversity and evolutionary potential in most forest tree species with large effective population sizes, it is, questionable whether this is also the best strategy for low-

density, small and isolated stands of light-demanding tree species. These forest stands are, however, common in many parts of Western Europe mainly as a consequence of past deforestation and fragmented forest ownership (Hyttinen 2001; Wiersum et al. 2005). Moreover, the transformation of monocultures into mixed and uneven-aged forest stands has reduced the number and size of monospecific forest patches (Maes et al. 2011). Studies that have extensively documented intergenerational transmission of tree genetic diversity in small-scale forest management systems (< 5 ha) are relatively rare, and most of them focused on conifers (Rajora et al. 2000; Kettle et al. 2007; Parker et al. 2001) or tropical tree species (Dayanandan 1999; Farwig et al. 2008). Furthermore, the few studies that have been conducted on broadleaved temperate tree species focused on elucidating patterns of pollen flow through genotyping seed material that was directly obtained from mother trees (Piotti et al. 2011; Butcher et al. 2005; Hoebee et al. 2007). Whereas such studies can identify pollen flow patterns that result in fruit set, they do not provide any information regarding the genetic composition of the progeny that effectively recruited into the population.

To better understand the processes that shape genetic diversity across generations in small and low-density forest stands, we analysed mating system and gene flow patterns in the common temperate broadleaved tree species pedunculate oak (*Quercus robur* L.) and compared genetic diversity between adult trees and naturally established seedling cohorts. Analyses were conducted in four closed canopy forest stands that were located in a matrix of agricultural land or stands of other tree species such as *Pinus sylvestris* L., *P. nigra* Arnold and *Fagus sylvatica* L. Since *Q. robur* is a light-demanding tree species characterized by a narrow recruitment window, only a recently established seedling cohort (1-3-year old) was found in the studied monospecific forest stands. This is not uncommon in European oak forests (Vera et al. 2006), and may influence the intergenerational transfer of genetic diversity. More specifically, we aimed:

- To assess to what extent genetic diversity is maintained between adults and recent naturally established seedling cohorts in small and low-density oak stands.
- To quantify mating patterns and within-stand gene flow.
- To compare fine-scale spatial genetic structure (SGS) between the two studied life-history stages.

3.3 Materials and Methods

3.3.1 Study species

Pedunculate oak (*Quercus robur L.*) is an indigenous tree species belonging to the white oak section (subgenus *Lepidobalanus*). Its natural range extends from Southern Scandinavia to the south of Europe, and eastwards to the Ural Mountains (Bary-Lenger & Nebout 1993). *Q. robur* occurs on a wide range of soils and is a keystone species of many European forest ecosystems. It is a monoecious and wind-pollinated species that has a highly outcrossing breeding system (selfing rate: 2 - 5%, Steinhoff 1993). The distinct male and female flowers are borne on staminate and pistillate inflorescences, respectively, which are carried on the same branches (Ducousso et al. 1993). Fertilization occurs 8 - 10 weeks after pollination, followed by rapid development of the acorns. These are dispersed during autumn by gravity, small rodents or birds, most notably the European Jay (*Garrulus glandarius* L.). A large number of acorns is produced during mast years, which may lead to a strongly increased propagule pressure and induce regeneration waves (Bary-Lenger & Nebout 1993). Acorns can germinate and establish under closed canopy cover. Seedlings, however, require high irradiance and thus strong forest canopy opening (30 - 40% crown projection area) for further growth and development (Vera et al. 2006; Bary-Lenger & Nebout 1993).

3.3.2 Study populations and sampling

In the summer of 2010 and the spring of 2012 we sampled four monospecific pedunculate oak stands in the central and eastern part of Flanders (Northern

Belgium), with population sizes ranging from 32 to 682 adult individuals, and densities ranging from 65 trees/ha to 195 trees/ha (Table 3.1). The investigated forest stands were part of old forests, which occur on the historical De Ferraris topographical maps (1772 - 1779) and which all have a past history of forest fragmentation and tree planting (Tack et al. 1993). Research permits for all forests were provided by the Agency for Nature and Forests of the Flemish government. Stands were either located in a matrix of agricultural land or other tree species. They also differed in their degree of isolation from the nearest *Q. robur* stand (Table 3.1). Because the study sites were characterized by a closed canopy cover, only recently established seedling cohorts (1-3-year old) were present in these closed canopy forests. The seedlings sampled most likely originated from acorns produced during the years 2009 and 2011, which were mast years for oak in Flanders (Sioen & Roskams 2012).

Table 3.1. Characteristics of the 4 studied pedunculated oak stands.

Forest stand	Latitude (*N*)	Longitude (*E*)	Area (ha)	Population size	Density (trees/ha)	Plot size (ha)	Isolation (m)
Keffers	50°50'26''	4°42'00''	3.04	328	118	0.49	400
Vos	50°49'27''	4°39'33''	3.97	682	195	0.24	175
Hornebos	50°43'02''	5°15'34''	4.5	242	65	0.78	1000
Chartreuse	50°54'45''	4°46'25''	0.43	32	74	0.43	135

In the centre of each stand, one large circular plot containing 35 pedunculate oak adults was established, ranging in size from 0.24 - 0.78 ha. All adult trees in this plot were sampled in the summer of 2010. In the smallest population (Chartreuse) all adult trees (32) present were sampled. At the same time, a subplot of 20 x 20m was established in the centre of each large circular plot. We followed this subplot approach in order to (1) increase the probability of assigning offspring to parents in the parentage analysis, and (2) conduct analyses of fine-scale spatial genetic structure and mating system. 100 arbitrarily selected seedlings were sampled in these subplots (Figure 3.1). Since these 100 seedlings might be half- or even full-sibs and thus

pseudo-replicates, they were not used in further analyses comparing genetic diversity between the adult generation and progeny. To allow for comparisons of genetic diversity (A_r, H_o, H_e, F_{IS}, rare alleles) between generations, an additional 35 recently established *Q. robur* seedlings were arbitrarily selected and sampled in spring 2012, across the large circular plots where also the adult generation was sampled.

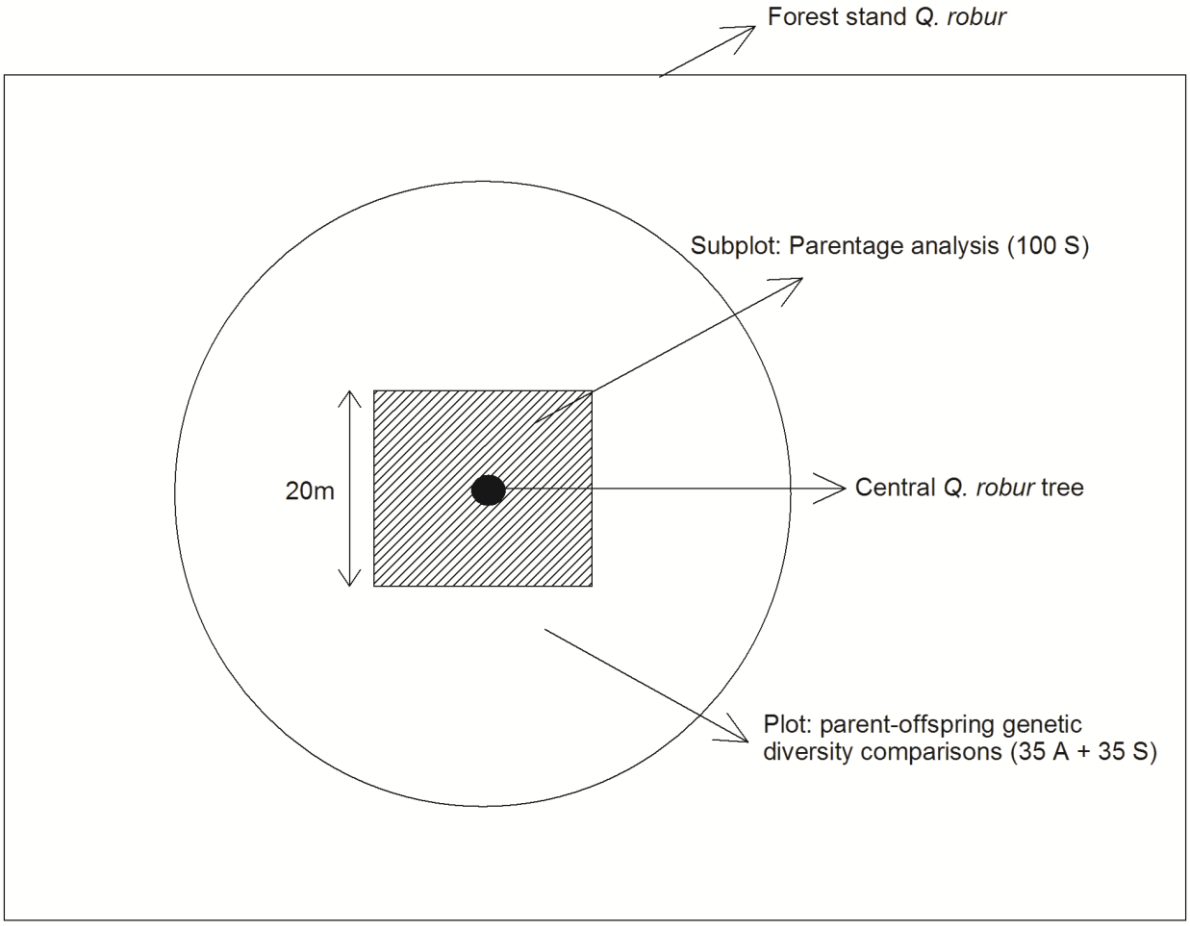

Figure 3.1. Sampling design of the study, with A: Adults and S: Seedlings.

To avoid that this seedling cohort contained various offspring from the same mother tree (half-sibs), a parentage analysis (CERVUS 3.0.3; Marshall et al. 1998) was conducted to assign these 35 seedlings to their mother trees. Seedlings that shared the same mother tree were not used in further analyses comparing genetic diversity between generations. Per stand we could obtain 25 seedlings, which all originated from different mother trees. All individuals were positioned up to the nearest cm using a FieldMap digital forest mapping system (IFER Ltd., Czech Republic),

connected with the Laser Rangefinder ForestPro and electronic compass MapStar Module II (both Laser Technology Inc., Colorado, USA). The sampled leaf tissue from adult trees and seedlings was dried on silica gel for DNA extraction.

3.3.3 Genetic markers

Dried leaf samples were ground up for DNA extraction using the Nucleospin Plant II kit (Macherey-Nagel). Ten nuclear microsatellite markers were selected for genetic analysis, six isolated from *Quercus petraea*: QpZAG9, QpZAG108, QpZAG46, QpZAG15, QpZAG110, QpZAG104 (Steinkellner et al. 1997), three from *Quercus macrocarpa*: MSQ4, MSQ13, MSQ16 (Dow et al. 1995; Dow & Ashley 1996) and one from *Quercus robur*: QrZAG112 (Kampfer et al. 1998). The microsatellites were located on seven different linkage groups (Barreneche et al. 1998). The Polymerase chain reaction amplifications were performed using the Multiplex PCR Master Mix kit (QIAGEN) and amplifications were carried out in a thermocycler programmed for the following PCR profiles: 15 min 94°C, followed by 30 cycles of 45 s 94°C, 45 s 50°C and 45 s 72°C and a final elongation step of 10 min at 72°C. Analysis of the amplified fragments was done with an SCE9610 genetic analyzer and GenoSpectrum™ software (version 3.0.0 beta) for the samples collected in 2010 and an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, USA) and GeneMapper sotware (version 4.1.) for the 2012 samples. Reference samples with known genotypes were used to align the genotypic data of 2012 to the dataset of 2010. The software Micro-Checker (Van Oosterhout et al. 2004) was used to detect genotyping errors resulting from the presence of null alleles, stutter peaks and large allele drop-out. A high null allele frequency (0.12 - 0.18) was consistently observed in one locus (MSQ16), which was removed from further analyses. The other microsatellites showed low null allele frequencies (< 0.05) in most populations. One microsatellite QpZAG108 showed stutter bands in a number of samples, leading to higher null allele frequencies (Appendix 3.1). For microsatellites with a high number of alleles (> 20 alleles), the total number of studied individuals (539 individuals) was sufficient to ensure a good representation of the allelic diversity (Appendix 3.2).

3.3.4 Data analysis

We calculated the following measures of genetic diversity: allelic richness (A_r), observed heterozygosity (H_o), Nei's unbiased expected heterozygosity (H_e) and Wright's inbreeding coefficient (F_{IS}), using GenAlEx (version 6.2, Peakall & Smouse 2006). We used the rarefaction approach described by El Mousadik and Petit (1996) to account for differences in sample size when calculating allelic richness. The percentage of rare alleles per study plot and per generation was calculated by dividing the number of low-frequency alleles (< 0.05) by the total number of alleles present in a stand. Differences in levels of A_r, H_o, H_e and F_{IS} between adults and progeny were analysed using the comparison among groups of samples test in Fstat (version 2.9.3.2; Goudet 1995) computing 1000 permutations. Estimates of effective population size (N_e) based on linkage disequilibrium were calculated for each forest stand using LDN_e (Waples & Do 2008). Only alleles with a minimum frequency of 0.05 (P_{crit}) were included in the analysis, since lower frequencies strongly increase the upward bias of N_e when sample size is smaller than 50 (Waples & Do 2008). Confidence intervals (95%) for N_e were obtained by jackknifing across loci. The presence of a substantial bias of N_e as a consequence of sample sizes much smaller than N_e was tested by sub-sampling the available sample at a range of sizes up to the full sample size (5 - 35 samples). N_e estimates were then plotted against these sample sizes (Appendix 3.3). Our sample size was appropriate to obtain correct estimates of N_e using LDN_e, as the estimates of N_e stabilized with increasing sample size (England et al. 2006). Other assumptions of the linkage disequilibrium method (closed populations, discrete generations) might not apply to many natural populations. However, we tried to obtain closed populations by locating stands in a matrix of agricultural land or other tree species. Furthermore N_e was calculated solely for the seedlings collected in the spring of 2012.

We performed a parentage analysis using CERVUS 3.0.3. (Marshall et al. 1998) based on multilocus genotypes of 100 seedlings, sampled in each 20 x 20m subplot, and 35 adults per study plot (Figure 3.1). This subplot was established in the centre of the adult plot to increase the probability of identifying both parent trees. High

exclusion probabilities for parental assignment (single parent exclusion ≥ 0.998 and parent pair exclusion ≥ 0.999) were obtained with the used set of SSR markers. The most likely parents and parent pairs were detected using LOD (log-likelihood ratio) scores (Marshall et al. 1998). LOD-score thresholds for parental assignment were determined by simulating 10,000 offspring. For all analyses, the proportion of mistyped loci and the error rate for likelihood calculations were set to 0.01 (default settings). The number of candidate parents used in the simulation studies ranged between 32 and 682 (all mature trees in the studied forest stands) with 100% to 5.1% of the candidate parents sampled. Parent-offspring matches were made based on the CERVUS 80% threshold level, and a simple exclusion analysis based on the multilocus genotypes of seedlings and adults (Nakanishi et al. 2005). Because previous studies on oak species indicated that acorn dispersal was more limited than pollen movement (Dow & Ashley 1996; Chybicki & Burczyk 2010), the following assumptions were made. First, offspring were assigned to both a mother and a father tree if the LOD-score threshold for parental pairs was exceeded by the parental pair with the highest LOD-score. In that case, the parent nearest to the seedling was designated as the mother tree. Second, when only the single parent LOD-score threshold was exceeded, the adult tree with the highest LOD was assumed to be the seed parent. After parental assignment, seed dispersal distances were calculated as the distance between established seedlings and their mother trees (distance from the stem), whereas pollen flow (inter-parent) distances could only be obtained if both parents were assigned to a seedling.

We investigated the effect of gene flow on the extent of fine-scale spatial genetic structure (SGS), since limited gene flow is considered the most important factor in the establishment of SGS (Vekemans & Hardy 2004; Jacquemyn et al. 2006). We assessed the extent of SGS in each population using Nason's kinship coefficient F_{ij} (Loiselle et al. 1995). Kinship coefficients were calculated both within (adult pairs and seedling pairs) and between generations (adult-seedling pairs). The multilocus genotypes of 100 seedlings, sampled in each 20 x 20m subplot, and 35 adults per study plot were used in these analyses. The extent of SGS between the adult and seedling cohort may provide insights into the role that gene dispersal from the

parent generation plays in shaping spatial genetic patterns within forest stands (Hamrick et al. 1993). Distance intervals were chosen according to recommendations of Hardy and Vekemans (2003), implying that at least 100 pairs of individuals were included within each distance class, and that 50 percent of the individuals were represented at least once in a pairwise comparison in each distance interval. Consequently, distance classes differed among generations and studied stands. To visualize SGS, kinship coefficients were plotted against distance classes to generate spatial genetic autocorrelograms. To verify the significance of kinship coefficients and S_p statistics, confidence intervals (95%) were obtained by permuting individuals among locations 10,000 times. All analyses were conducted using SPAGeDi 1.3 (Hardy & Vekemans 2002).

We conducted a mating system analysis using the program MLTR version 3.2 (Ritland 2002). MLTR was applied to a subset of progeny that was categorically assigned to their mother tree in the parentage analysis. Only families composed of more than 5 seedlings per mother tree were included in the analyses. Maximum-likelihood estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates were based on mixed mating models, and the difference between both parameters (t_m - t_s) was calculated to determine biparental inbreeding. Correlated paternity (r_p) was estimated as the probability that two seedlings drawn at random from the same mother tree shared the same father tree. The effective number of pollen donors per mother tree was estimated as $1/r_p$. Confidence intervals (95%) for the estimated mating system parameters were obtained by re-sampling families within populations using 1000 bootstraps. These 95% confidence intervals were used to evaluate whether estimates were significantly different from each other and from zero.

In addition, the TWOGENER method was used to characterize correlated mating (Smouse et al. 2001), as implemented in the software package POLDISP 1.0c (Robledo-Arnuncio et al. 2007). Contrary to the MLTR method, the TWOGENER method account for spatial information and does not consider selfing and immigration (Gauzere et al. 2013). The molecular differentiation between global pollen pools Φ_{FT}, and a corresponding variance estimator s^2_{Φ}, was calculated for the

above TWOGENER-dataset following Smouse et al. (2001). The parameters $2\Phi_{FT}$ and $1/2\Phi_{FT}$ were used as an alternative estimator for correlated paternity and the effective number of pollen donors. Confidence intervals (95%) around Φ_{FT} were based on the estimated standard deviation (s_{Φ}).

3.4 Results

For the 539 studied individuals, the total number of alleles per locus ranged from 11 (QpZAG9) to 36 (QpZAG104), with an average of 21.6 (± 2.7) (means are followed by SE in parentheses). The pooled sample of naturally established seedlings across all 4 forest stands showed a significantly higher (exact test, 1000 permutations, $p = 0.043$) allelic richness (A_r) in adult trees 11.5 (± 0.2) than in the seedling cohort 10.7 (± 0.2) (Table 3.2). Higher levels of A_r in adults compared with seedlings were also detected for each population separately. However, the standard errors of A_r were large, suggesting that the observed trends were rather weak. Contrary to the mean A_r across populations, observed heterozygosity (H_o), expected heterozygosity (H_e) and inbreeding coefficient (F_{IS}) did not differ between the adult and progeny cohort (exact test, 1000 permutations, $p > 0.05$) (Table 3.2). The percentage of rare alleles clearly decreased (28.4%) across generations in the forest stand Vos, which exhibited the highest proportion of rare alleles in the adult generation (Figure 3.2). In all

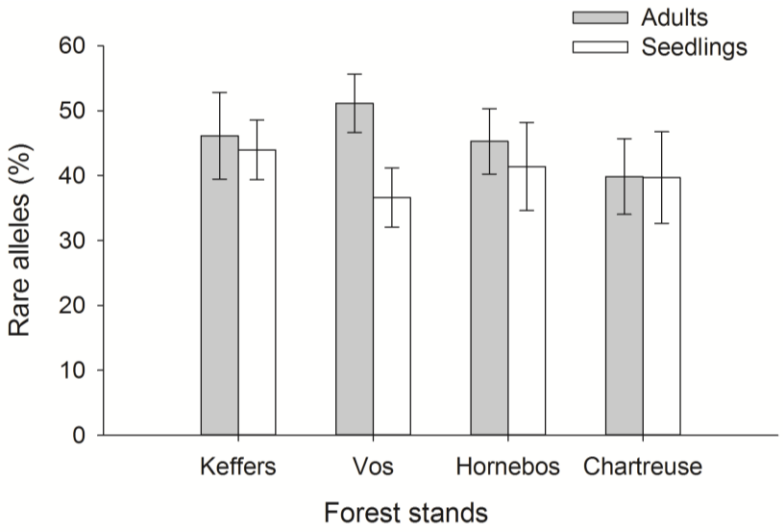

Figure 3.2. Percentage of rare alleles ± standard errors compared between adults and seedlings present in the four studied forest stands.

stands, the effective population size (20 - 60 individuals) was smaller than the census population size (32 - 682 individuals) and decreased with declining census population size (Table 3.2).

3.4.1 Parentage analysis

Of the 100 seedlings sampled in each 20 x 20 m subplot, a high percentage (81 - 93%) could be unambiguously assigned to a mother tree from within the study plot (Appendix 3.4). In the stands Chartreuse, Keffers and Vos, 90% of the assigned seedlings were growing within a 10 m radius from the stem of their mother tree (Appendix 3.5). Seed dispersal distances were small in these stands, with average values ranging from 4.2 m (± 0.03) to 4.9 m (± 0.03). Higher levels of seed dispersal were found in Hornebos, where an average distance of 15.4 m (± 1.2) was observed, with 75% of the seedlings present within 20 m of their mother. The number of seedlings assigned to a pollen donor differed between the sampled forest stands (Appendix 3.4). Pollen flow from outside the study plot was extensive in the stands Keffers and Vos, where 67% and 79% of the seedlings resulted from pollination by pollen from trees outside the study plot respectively. In Chartreuse and Hornebos, however, most seedlings (68% and 60%) originated from fathers within the plot.

3.4.2 Fine-scale spatial genetic structure (SGS)

No significant spatial genetic structure was observed for the parent trees (results not shown), whereas seedlings and seedling-adult pairs showed significant spatial genetic structure (Appendix 3.6). Kinship coefficients peaked outside the 95% confidence interval at the smallest distance intervals, followed by a steep decline at greater distance classes (Figure 3.3). In Chartreuse, Keffers and Vos, between-generation SGS was detected up to a distance of 8 - 10 m (Figure 3.3), resulting in significant *Sp*-statistics (95% CI excludes zero) that varied between 0.016 and 0.019. Hornebos, however, showed significant positive values of F_{ij} that occurred at larger distances (up to 14 m, $Sp = 0.008$). Negative F_{ij}-estimates were observed at 8 and 13 m in Vos and Keffers (Figure 3.3), indicating that a smaller number of alleles was shared between individuals than expected by chance.

Table 3.2: Estimates of effective population size (± confidence intervals) and genetic diversity measures (± standard errors) for adults and progeny from four *Q. robur* populations based on 9 microsatellite loci.

	N_e	A_r*		H_o		H_e		F_{IS}	
	(P_{crit} = 0.05)	Adult	Progeny	Adult	Progeny	Adult	Progeny	Adult	Progeny
Keffers	45.7 (29.3-86.1)	12.0 (1.2)	11.1 (1.1)	0.772 (.036)	0.813 (.036)	0.825 (.028)	0.824 (.023)	0.066 (.029)	0.014 (.051)
Vos	58.4 (32.3-168.2)	11.8 (1.2)	10.4 (1.1)	0.819 (.041)	0.786 (.034)	0.823 (.033)	0.822 (.027)	0.006 (.033)	0.045 (.028)
Hornebos	34.6 (23.8-55.6)	11.1 (1.2)	10.8 (1.1)	0.793 (.039)	0.813 (.047)	0.818 (.028)	0.817 (.031)	0.031 (.039)	0.005 (.034)
Chartreuse	22.6 (14.6-38.0)	11.1 (1.4)	10.4 (1.4)	0.808 (.025)	0.805 (.051)	0.818 (.033)	0.805 (.031)	0.013 (.020)	-0.001 (.042)
Mean**	40.3	11.5 (0.2) **a**	10.7 (0.2) **b**	0.798 (.010) **a**	0.804 (.010) **a**	0.821 (.002) **a**	0.817 (.004) **a**	0.029 (.013) **a**	0.016 (.010) **a**

N_e, *effective population size with* P_{crit} *the minimum frequency for alleles to be included in the analysis;* A_r, *allelic richness;* H_o, *observed heterozygosity;* H_e, *expected heterozygosity;* F_{IS}, *inbreeding coefficient;*

**allelic richness based on a minimum sample size of 25 individuals*

***means across populations followed by the same letter are not significantly different at the p < 0.05 level (1000 permutations, Fstat)*

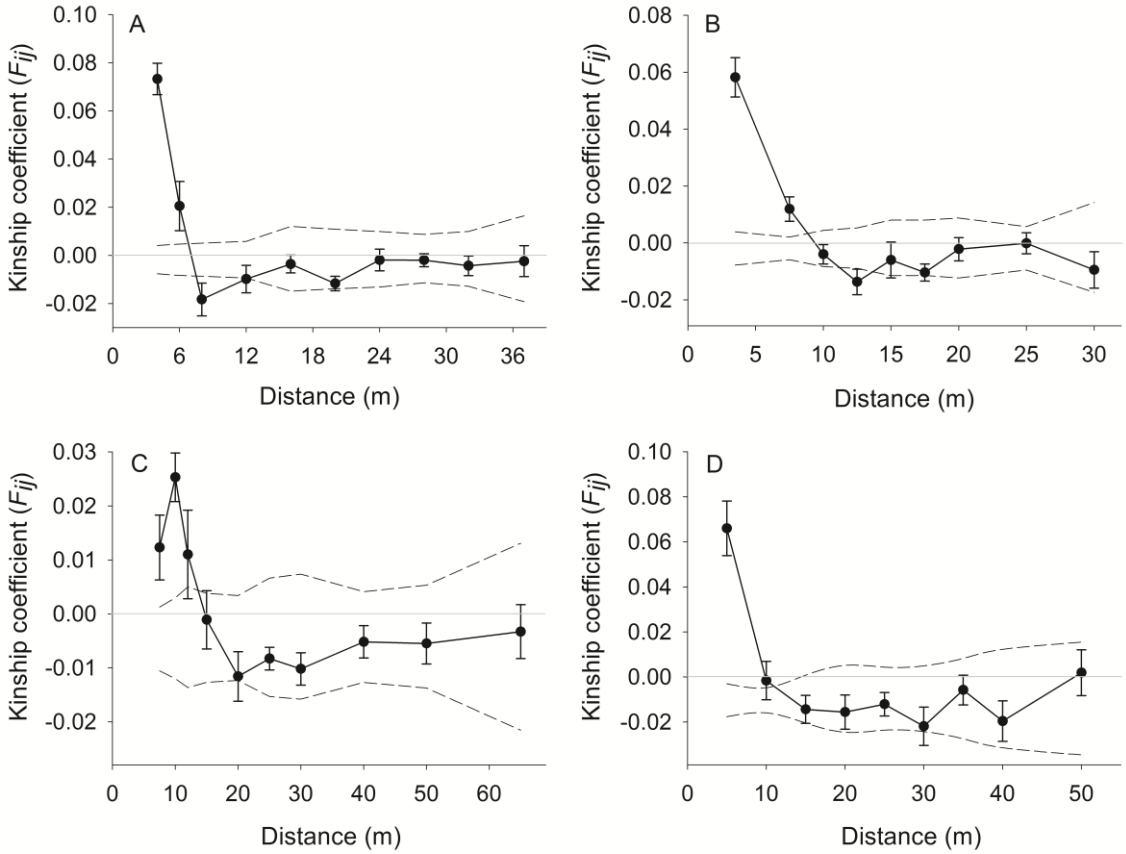

Figure 3.3. Auto-correlograms of Nason's kinship coefficients (F_{ij}) ± standard deviations for adult-seedling pairs in the four studied forest stands: Keffers (A), Vos (B), Hornebos (C), Chartreuse (D). The dashed lines indicate upper and lower 95% confidence intervals (10,000 permutations).

3.4.3 Mating system analysis

High levels of multilocus outcrossing were observed for the four studied populations (t_m = 0.984 - 1.000), supporting the outcrossing nature of *Q. robur* (Table 3.3). Estimates of biparental inbreeding were in general low across all study plots (t_m - t_s = 0.029 - 0.063), but they were significantly higher than zero (95% CI excludes zero) in the forest stands Chartreuse and Vos. The presence of significant biparental inbreeding in these stands was most likely the result of single-locus outcrossing rates, which were significantly lower than one (95% CI excludes one). The 95% confidence intervals of all estimates of correlated paternity (r_p, MLTR and $2\Phi_{FT}$, TWOGENER) did not include zero, suggesting the presence of full sibs within the seedling cohort. Using Ritland's MLTR model, we found significantly higher levels of

correlated paternity in the stand Chartreuse (r_p = 0.135) compared to the other forest stands (non-overlapping 95% CI, r_p = 0.036 - 0.063) (Figure 3.4). The values obtained with the TWOGENER method showed a similar tendency, although somewhat lower. In this case, the correlated paternity of Chartreuse differed only significantly with the stands Keffers and Vos (Figure 3.4). For both models, the average ''paternal mating pool'', the estimated number of pollen donors per mother tree ($1/r_p$, MLTR and $1/2\Phi_{FT}$, TWOGENER) was low in Chartreuse (respectively 7.4 and 11.2 fathers), whereas in the remaining populations ca. 2 to 4 times more fathers per mother trees were estimated (Table 3.3).

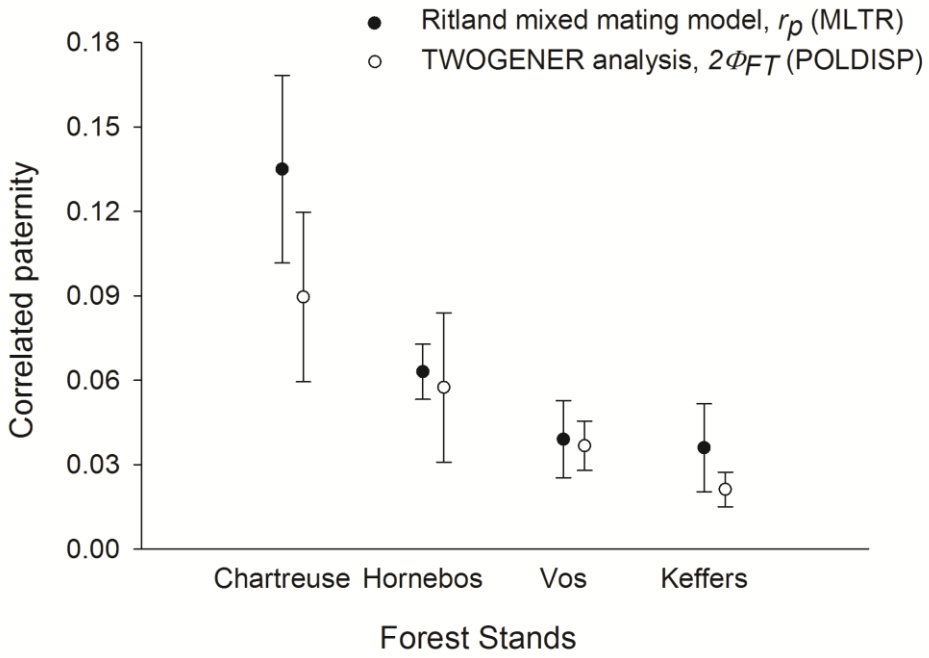

Figure 3.4. Estimators of correlated paternity (r_p and $2\Phi_{FT}$) and 95% bootstrap confidence intervals for the four studied *Q. robur* stands.

Table 3.3 Mating system estimates and their standard errors (in parentheses) for the four studied *Q. robur* stands.

Forest stand	t_m	t_s	$t_m - t_s$	Correlated paternity estimators		# Pollen donors	
				r_p	$2\Phi_{FT}$	$1/r_p$	$1/2\Phi_{FT}$
Keffers	0.987 (0.009)	0.950 (0.020)*	0.037 (0.022)	0.036 (0.008)*	0.021 (0.003)*	27.8	47.3
Vos	1.000 (0.000)	0.960 (0.009)*	0.040 (0.009)*	0.039 (0.007)*	0.037 (0.004)*	25.6	27.3
Hornebos	0.984 (0.015)	0.956 (0.025)	0.029 (0.018)	0.063 (0.005)*	0.057 (0.014)*	15.9	17.4
Chartreuse	1.000 (0.000)	0.937 (0.017)*	0.063 (0.017)*	0. 135 (0.017)*	0.090 (0.015)*	7.40	11.2

t_m, *multilocus outcrossing rate;* t_s, *single-locus outcrossing rate;* $t_m - t_s$, *biparental inbreeding;* r_p, *correlated paternity estimated with Ritland's mixed mating model (2002);*Φ_{FT}, *Paternity differentiation between families TWOGENER method;* $1/r_p$ *and* $1/2\Phi_{FT}$, *Effective number of pollen donors from MLTR and TWOGENER respectively.*

**Significantly less than 1 for* t_m *and* t_s, *and significantly greater than zero for* $t_m - t_s$, r_p *and* $2\Phi_{FT}$ *(*$p < 0.05$*)*

3.5 Discussion

3.5.1 Genetic diversity of adults and seedlings

No clear differences (large standard errors) between the genetic diversity measures of the offspring cohort and adult generation were found in the small and low-density forest stands that were studied. Only the naturally established seedlings in the stand Vos showed a strong decrease (-28.4%) of the number of rare alleles. Such unpredictable fluctuations in allele frequencies can likely be attributed to genetic drift, since drift may rapidly remove low frequency alleles (Young et al. 1996; Ellstrand & Elam 1993). However the overall effect of genetic drift on allelic richness (A_r) was rather limited, and only when all stands were pooled, the adult cohort showed a slightly but significantly higher allelic richness than the recently established (1-3-year old) seedling cohort. This may indicate that effective population sizes (N_e) were large enough to avoid strong losses of genetic diversity during reproduction (Lande 1995; Franklin & Frankham 1998). The non-significant changes across generations that were obtained for observed heterozygosity (H_o) and the inbreeding coefficient (F_{IS}) support this finding, and suggest that inbreeding was low or negligible. However, F_{IS} and H_o are less sensitive indicators of low N_e than A_r, and may require extended periods of low N_e before changes in H_o or F_{IS} become detectable (Young et al. 1996). Furthermore, because we only sampled recently established seedling cohorts (1-3-year old) and because adult trees may reproduce during many decennia, the above results need to be interpreted with caution as they may have underestimated the total genetic diversity that could be transferred during natural regeneration of the stands. The genetic composition of seedlings may vary across years, due to stochasticity and variation in environmental factors that affect mating system, gene flow and early mortality (Petit & Hampe 2006). Although the recruitment window of light-demanding tree species such as *Q. robur* is restricted in time (mast years) and space (light requirement), it would be beneficial to sample seedlings over a larger number of seasons to get a better idea of seasonal fluctuations in gene flow and genetic composition of the seedlings.

3.5.2 Contemporary gene flow

The high outcrossing rates observed in all stands (Table 3.3) support the nearly complete self-incompatibility system in the genus *Quercus*, which can be attributed to the gametophytic control of the pollen tube growth in the style (Ducousso et al. 1993). Artificial pollination experiments conducted by Aas (1991) and Steinhoff (1993) revealed that most of the studied oak individuals possess this gametophytic self-incompatibility system, through which selfing is rarely distributed over oak populations. The selfing rates found here (s = 0.7%) were lower than the estimates reported by Steinhoff (s = 1.8%, 1993). However, contrary to Steinhoff (1993), who analysed control-pollinated seeds, we measured the realized outcrossing rate at the seedling stage, where early selection and random drift effects might have eliminated less fit homozygotes (Honnay et al. 2008). Despite the predominantly outcrossing nature of *Q. robur*, and the small proportion of biparental inbreeding that was observed (t_m - t_s = 0.029 - 0.063), significant spatial genetic structure (SGS) was found within the seedling cohort. In contrast, the adult generation did not show a significant SGS in all forest stands, which could be explained both by tree planting in the past and by the spatial scale of the adult plots which was insufficiently large (only 32 - 35 trees) to span the SGS of the adult generation (Dostálek et al. 2011). The SGS in the seedling cohort was observed at the shortest distance classes (< 10 m) and can most likely be attributed to half- and full-sibs typically growing beneath the canopy of their mother trees, as an important fraction of acorns falls beneath the canopy of the mother tree (Streiff et al. 1998). Indeed, we found that most seedlings (81 - 93%) originated from mother trees within the study plot (Appendix 3.4 & 3.5). Apart from the limited seed dispersal, the low tree density may also have contributed to the observed SGS. Overlapping seed shadows, which are more likely to occur in high-density forests, may decrease levels of spatial genetic structure (Nakanishi et al. 2005). In the low-density forest stands (Chatreuse and Hornebos) we found significant SGS which occurred at larger distances compared to the stands with higher tree densities (Vos and Keffers). Although restricted seed dispersal is a general characteristic of oak species (Dow & Ashley 1996; Chybicki & Burczyk 2010),

some studies have indicated that dispersal by birds could strongly increase the long-distance component of the dispersal kernel (Gómez 2003; Moran & Clark 2011). Our estimates of the average seed dispersal distance (4.9 - 15.4 m) were similar to dispersal distances found for *Quercus robur* and *Q. petraea* by Chybicki & Burczyk (2010) (8.8 - 15.6 m). Furthermore, the higher levels of seed dispersal (15.4 m) that were found in Hornebos may be partly caused by the larger mean tree height and larger crown sizes in this stand (Cousens & Rawlinson 2001).

In all study plots, relatively low seed immigration rates (7 - 19%) were observed, confirming that gene flow was mainly mediated by pollen. On the other hand, the proportion of pollen originating from outside the study plots was much higher (32 - 79%). High percentages of immigrant pollen are not necessarily due to long distance pollination events, but may also result from pollination of adult trees from outside the study plot that were located within the oak stand. High percentages of out-of-plot pollen immigration rates are known for *Quercus* species, usually ranging between 50 - 70% (Chybicki & Burczyk 2010; Nakanishi et al. 2005). However, Valbuena-Carabana et al. (2005) obtained lower pollen immigration rates (< 40%) for *Q petraea* and *Q. pyrenaica*. The differences in pollen immigration rates between study plots suggest that stand characteristics in small scale forestry systems can have a major impact on pollen flow patterns. In the case of small or low-density stands, few trees may contribute pollen (Smouse & Sork 2004; Breed et al. 2013b). Indeed, the smallest stand Chartreuse (32 individuals), which was also characterized by a low tree density, showed a significantly higher correlated paternity, and thus a lower estimated number of pollen donors per mother tree than the other stands (Figure 3.4). Similar results were found in the wind-pollinated tree *Pinus sylvestris* by Robledo-Arnuncio et al. (2004). In this study, seed trees of the smallest stand (36 individuals) showed 100-fold higher estimates of correlated paternity than the larger stands (> 10^5 individuals).

Contrary to Robledo-Arnuncio et al. (2004) who did not find a clear effect of tree density on correlated paternity, tree density may have influenced pollen flow in our study. For example, the medium-sized forest stands Hornebos (242 individuals)

and Keffers (328 individuals), which differed in tree densities (65 trees/ha and 118 trees/ha respectively), showed differences in correlated paternity (0.063 versus 0.036) and pollen immigration rates (40% versus 67%). These results are consistent with earlier studies in other *Quercus* species (*Q. humboldtii*, *Q. alba*, *Q. lobata*), which showed a positive relationship between the density of a forest stand and the estimated number of pollen sources contributing to the offspring (Sork et al. 2002; Fernández-Manjarrés & Sork 2005). However, although the forest stand Vos exhibited the largest population size (682 individuals) and highest tree density (195 trees/ha), the estimates of correlated paternity were similar to the ones found in the stand Keffers (328 trees, 118 trees/ha). This may indicate that in dense oak populations local mating may play a major role, together with high rates of pollen immigration (Chybicki & Burczyk 2010; Fernández-Manjarrés et al. 2006).

3.5.3 Perspectives for maintaining genetic diversity in small oak stands

In the literature, the 50 - 500 conservation "rule" is often cited as a general guidance to maintain viable populations among taxa (Franklin 1980). According to this guideline, a minimum effective population size (N_e) of 50 individuals is necessary to prevent populations against the immediate effects of inbreeding depression, whereas effective population sizes of at least 500 individuals have been proposed to be necessary for the conservation of the evolutionary potential in the long term (Franklin & Frankham 1998). However, based on the inbreeding depressions that have been reported in laboratory populations and based on more recent alternative quantitative genetic theory, the 50 - 500 rule should at least be doubled (100 - 1000 rule) to avoid inbreeding and to limit the total fitness loss (< 10%) in the short term (5 generations) and for maintaining the evolutionary potential of natural populations (Frankham et al. 2014).

Although we obtained low N_e estimates in all studied stands (22.6 - 58.4 individuals), no selfing and only very marginal estimates of biparental inbreeding were observed. However, higher estimates of correlated paternity were found in stands with low N_e. Consequently, stronger effects of biparental inbreeding in subsequent generations may be expected, as high levels of correlated paternity will

increase the probability of mating among half- and full-sibs (Young & Brown 1999). In addition, reduced pollen diversity may have fitness costs on future offspring generations independently of inbreeding, as a lower diversity of pollen can increase the number of recessive deleterious alleles within the paternal mating pool and decrease pollen competition (Breed et al. 2012). Although each population separately showed the tendency of lower A_r in the offspring, no strong differences between the genetic diversity measures of the offspring cohort and adult generation were found. This supports the findings that genetic drift and inbreeding were minimal or may not yet have been detectable in the studied stands and indicates that other factors may influence or mitigate the genetic consequences of small population size (Williamson-Natesan 2005). An important factor that may increase the effective population size in natural regenerating oak forests is extensive pollen flow (Dow & Ashley 1998). The high (> 30%) pollen immigration rates that were observed in our study, may have counteracted the negative genetic consequences associated with small forest stands. In oak species, pollen may travel up to several kilometres, maintaining high levels of genetic diversity within oak stands (Dow & Ashley 1998; Buschbom et al. 2011). However, UV light may degrade oak pollen (Schueler et al. 2005) and local matings may limit effective pollen dispersal distances (Ducousso et al. 1993), which can eventually lead to population subdivision (Sork et al. 2002; Fernández-M & Sork 2005). To allow gene inflow from neighbouring stands, limiting isolation between monospecific stands may be more important than previously thought, even in highly outcrossing tree species. To maintain and increase genetic diversity in small scale forestry systems, natural regeneration could be supplemented by planting and sowing using genetically diverse reproductive material (Broadhurst et al. 2008; Vander Mijnsbrugge et al. 2010). Furthermore, the practice of transferring seeds or seedlings from different provenance regions may broaden the evolutionary potential to adapt to future environmental changes (Broadhurst et al. 2008; Breed et al. 2013a).

Chapter 4.

Tree density and population size affect pollen flow and mating patterns in small fragmented forest stands of pedunculate oak (*Quercus robur* L.)

Adapted from:

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4.1 Abstract

The loss and fragmentation of forests have resulted in landscapes with isolated forest patches embedded in an agricultural or urban landscape matrix. At the same time, stand characteristics such as tree population size and tree density have been strongly modified as a result of anthropogenic activities. Because increased geographical isolation can impede gene flow among forest fragments, and because decreased population size and tree density may reduce the number of local mating partners, this can be expected to lower the opportunity to trees for outcrossing, and to result in increased inbreeding, negatively impacting the viability of the tree populations. In this study, we examined eight stands of the wind-pollinated tree species *Q. robur* that strongly differed in population size, tree density and spatial isolation. In the centre of each stand, adult leaves and seeds were collected in a circular plot, in which we evaluated the diversity and differentiation of the local pollen pool, and examined mating patterns. Most forest stands showed high proportions of out-of-plot pollen flow (range: 0.24 - 0.77), which were positively correlated with the number and density of adult trees within the stands. Despite high outcrossing rates (> 0.998), seeds within seed families were stronger related than what could be expected under panmixia, which could be attributed to small but significant levels of correlated paternity (0.018 - 0.107) and biparental inbreeding (0.025 - 0.118) within the study plots. Next to increased coancestry coefficients, deviations from random mating also resulted in significant pollen pool differentiation (0.008 - 0.059) among seed parents. We also found that stand population size and tree density were significantly correlated to the relatedness of the seedlings and the degree of pollen differentiation within the study plots. These results suggest that, in small and isolated low density forest stands, reduced mate availability may decrease pollen pool diversity, increasing the likelihood of consanguineous mating and pollen pool differentiation in the next generations. We conclude that preserving high levels of pollen flow within and between forest fragments may be more important in wind-pollinated tree species than what was previously thought.

4.2 Introduction

In many parts of the world, once large and continuous forests have been replaced by a mosaic of forest fragments embedded in an agricultural or urban matrix (Riitters et al. 2000). Stand characteristics such as tree population size and tree density can also be strongly modified following anthropogenic disturbance, which in turn may have important consequences for tree pollen flow patterns and mating (Ouborg et al. 2006; Eckert et al. 2010). Increased geographical isolation can impede gene flow among forest fragments, whereas decreased population size and tree density reduce the number of local mating partners within forest stands (Smouse & Sork 2004; Breed et al. 2013b). Altogether, this can be expected to lower the opportunity for trees to outcrossing and can result in increased inbreeding, which may in the long term negatively impact on the genetic diversity and viability of fragmented tree populations (Ellstrand & Elam 1993; Young et al. 1996; Vranckx et al. 2012).

Although there is evidence of pollen limitation in tree species (Sork et al. 2002; Fernández-Manjarrés & Sork 2005; Jump & Penuelas 2006; Breed et al. 2012), the extent of it remains highly debated in forest conservation genetics, resulting in what is known as the "paradox of forest fragmentation genetics" (Kramer et al. 2008). Some studies have indeed not found a reduction of the diversity of the pollen pool following forest fragmentation (O'Connell et al. 2007), or have even demonstrated an increased diversity (Bacles et al. 2005). The latter can be explained by the smaller size and the lower tree density of forest fragments. When there are fewer nearby trees available as a pollen source, pollen competition between distant and nearby trees will be lower. As a result, the proportion of pollen from distant (unrelated) trees increases, and consequently the average realized pollination distance will be larger (Robledo-Arnuncio et al. 2004; Bacles & Ennos 2008; Wang et al. 2010). Moreover, a reduction in tree density and opening up of the landscape may improve air movements within and between forests (Okubo & Levin 1989). This can promote pollen flow, especially in wind-pollinated tree species, which produce large amounts of small and lightweight pollen (Young et al. 1993; Dyer & Sork 2001; Bacles et al. 2005). Such enhanced realized pollen dispersal can ultimately increase the effective

population size and thus mitigate the expected negative impacts of forest fragmentation on genetic diversity (Sork & Smouse 2006; Bacles & Jump 2011).

However, when the number of adult trees within a forest stand decreases too strongly, the larger average effective pollination distances may not totally compensate for the reduction in population size (Sork et al. 2002; Sork & Smouse 2006). This may not only affect the fitness of future offspring cohorts through increased selfing and biparental inbreeding (Sork et al. 2002; Breed et al. 2013b), but smaller pollen loads may also reduce the diversity of the local pollen pool, through which the proportion of recessive deleterious alleles within the paternal mating pool increases (Goubitz et al. 2002; Breed et al. 2012). In addition, supplementation of the local pollen pool with high rates of out-of-plot pollen flow is not necessarily a safeguard against genetic bottlenecks, as the number and diversity of pollen sources from outside a forest fragment that contribute to the local pollen pool can be small (Sork & Smouse 2006). Fernández-Manjarrés and Sork (2005) found higher pollen immigration rates of *Quercus humboldtii* Bonpl. in small forest fragments, but genetic diversity in the seedling cohort was significantly lower, suggesting that only a small effective number of pollen donors contributed to the pollen pool of the fragmented forest stands.

A better understanding of the combined effects of reduced adult numbers and average pollination distances on the local pollen pool diversity is indispensable for understanding the genetic consequences of forest fragmentation (Sork & Smouse 2006). In this study, we investigated the effects of different stand characteristics on mating patterns and pollen-mediated gene flow in forest stands of the common temperate broadleaved tree species pedunculate oak (*Quercus robur* L.). Studies that have documented the relationship between a range of population parameters (population size, tree density and isolation) on the one side and mating and pollen flow patterns on the other in more than four forest stands are relatively rare (Robledo-Arnuncio et al. 2004; Byrne et al. 2007; Breed et al. 2013b; Ismail et al. 2012; Llorens et al. 2012), and we are unaware of such studies in temperate deciduous tree species. We selected eight forest stands that strongly differed in population size, tree

density and isolation and which were located in a matrix of agricultural land or of stands of different tree species (*Pinus sylvestris* L. or *Fagus sylvatica* L.). The oak stands are representative for the typically strongly fragmented forest landscape of Northern Belgium (most fragments < 5ha), but also for other densely populated and highly urbanized regions in Europe (Vandekerkhove 2013). More specifically, we aimed at:

- Evaluating pollen competition between distant and nearby trees, by comparing the proportion of seeds that originated from fathers within the established study plots with pollen-mediated gene flow from outside the study plots, based on a traditional (maximum likelihood) paternity analysis (Marshall et al. 1998).
- Examining local pollen donor diversity and mating system parameters using both a correlated-mating model (Ritland 2002) and a two-generation analysis of pollen flow within forest stands (Smouse et al. 2001).
- Clarifying the role of stand characteristics such as population size, tree density, spatial isolation and the type of the landscape matrix on local pollen pool diversity and mating system parameters.

4.3 Materials and methods

4.3.1 Study species

Pedunculate oak (*Quercus robur* L.) is a common tree species of many European deciduous lowland forests. It occurs from sub-Mediterranean Europe to southern Scandinavia, and eastwards to the Ural Mountains (Bary-Lenger & Nebout 1993). Although *Q. robur* has a wide ecological range, it grows best on neutral, well-drained soils. *Q. robur* is monoecious and possesses a nearly complete gametophytic self-incompatibility system. As a result, selfing rates are low (2 - 5%, Steinhoff 1993). Separate staminate and pistillate flowers that are carried on the same branches further contribute to the low selfing rates. Trees flower generally from April to the end of May in Belgium (Ducousso et al. 1993). The pollen of pedunculate oak is one of the smallest (26 - 29 μm, Rushton 1976) and lightest among wind-pollinated

woody plant species, enhancing the potential for long-distance pollen flow (Chybicki & Burczyk 2010). Eight to ten weeks after pollination, fertilization and rapid fruit (acorn) development occur. Acorns of *Q. robur* are primarily dispersed beneath the canopy of the mother tree by gravity (Streiff et al. 1998), although dispersal by rodents and especially birds (most notably the European Jay (*Garrulus glandarius* L.) is known to substantially increase the long-distance potential component of the seed dispersal kernel (Gómez 2003; Moran & Clark 2011).

4.3.2 Study sites and sampling

We sampled eight pedunculate oak stands across the Flanders region of Belgium. *Q. robur* was the dominant tree species in these forest stands, and the understory was mainly composed of *Q. robur* seedlings, *Rubus* species and small trees of *Acer pseudoplatanus* and *Corylus avellana*. Stand characteristics were determined from forest registers (Bosprog-Bosdat v. 2.33, ANB 2006), using GIS data layers of the studied forest stands and from measurements with a FieldMap digital forest mapping system (IFER Ltd., Czech Republic). Stands differed in size (range: 32 - 704 individuals), tree density (range: 52 - 195 individuals/ha) and degree of isolation (distance from the nearest *Q. robur* stand, range: 135 - 5000m) (Table 4.1). The sampled stands were either located in a matrix of forest composed of other tree species, or in a matrix of agricultural land. In the centre of each *Q. robur* stand we established a circular plot containing ca. 35 adult trees (Table 4.1). Trees were mapped to the nearest cm using a FieldMap digital forest mapping system (IFER Ltd., Czech Republic) and from each sampled tree a leaf was sampled for microsatellite analysis. Within each circular plot, five seed traps (1.5 m^2 each) were randomly placed, with each seed trap positioned under the canopy of a single mother tree. From each seed trap ten acorns were randomly collected in the Autumn of 2011. Since 2011 was a mast year for pedunculate oak in Flanders (Sioen & Roskams 2012), acorn production was high and most seed traps contained plenty of acorns. The collected acorns were stored at 5°C for 4 weeks to increase the percentage and synchronization of seed germination (Manzanera et al. 1993). After cold storage the acorns were sown in seed trays containing commercial soil (20% organic matter,

pH = 6, EC = 750 μS cm^{-1}, N:P:K 14:16:18 1 kg m^{-3}), and grown in a greenhouse under controlled environmental conditions (12/12h day/night light regime, 20°C). One month after seedlings emerged, leaves were taken from each individual for DNA extraction.

4.3.3 Microsatellite analyses

Leaf samples from both adult trees and seedlings were stored on silica gel prior to DNA extraction. 200 mg dried leaf tissue was homogenized to a fine powder followed by DNA extraction with the Nucleospin Plant II kit (Macherey-Nagel). Ten microsatellites were used, which were isolated from *Q. robur*: QrZAG112 (Kampfer et al. 1998), *Quercus. petraea*: QpZAG9, QpZAG108, QpZAG46, QpZAG15, QpZAG110, QpZAG104 (Steinkellner et al. 1997) and *Quercus macrocarpa*: MSQ4, MSQ13, MSQ16 (Dow et al. 1995; Dow & Ashley 1996). Microsatellites were amplified in three multiplex PCRs using the Multiplex PCR Master Mix kit (QIAGEN). PCR cycling was carried out in a thermocycler programmed as follows: 30 rounds of 45 s 94 °C, 45 s 50 °C and 45 s 72 °C and a final extension step of 10 min at 72 °C. The PCR products were analyzed on an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, USA). Microsatellite alleles were visualized and scored using GeneMapper v. 4.1. We checked the microsatellites for possible genotyping errors such as null alleles, stutter peaks and large allele drop-out, using the software Micro-Checker (Van Oosterhout et al. 2004). The frequencies of null alleles were estimated using GENEPOP 4.0 (Rousset 2008). One locus (MSQ16) showed consistently high null allele frequencies (0.05 - 0.15), and was therefore removed from further analyses. In most forest stands, the 9 remaining microsatellites showed low null allele frequencies (< 0.05) (Appendix 4.1).

Table 4.1. Characteristics of the eight studied pedunculate oak stands.

Forest stand	Latitude (*N*)	Longitude (*E*)	Area (ha)	Population size	Density (trees/ha)	Isolation (m)	Landscape matrix	Plot size (ha)	Sampled adults	Sampled seedlings	Number of families[a]
Keffers	50°50'26''	4°42'00''	3.04	328	118	400	Forest	0.49	35	50	4
Vos	50°49'27''	4°39'33''	3.97	682	195	175	Forest	0.24	35	50	5
Hornebos	50°43'02''	5°15'34''	4.50	242	65	1000	Agriculture	0.78	37	50	4
Chartreuse	50°54'45''	4°46'25''	0.43	32	74	135	Forest	0.43	32	49	5
Overheide	51°25'17''	5° 03'16''	1.05	139	132	360	Forest	0.28	36	48	5
Hoge Vijvers	51°21'28''	5° 06'27''	3.80	704	186	240	Forest	0.19	36	49	5
Meikensbos	50°58'40''	3°22'52''	1.64	171	104	> 2 km	Agriculture	0.33	35	50	5
Egemse Veldekens	51°02'02''	3°16'59''	6.24	324	52	> 5 km	Agriculture	0.88	35	50	5

[a]Number of families used for the estimation of gene flow and mating system parameters in the 8 stands.

4.3.4 Data analysis

First, we calculated the following population genetic diversity measures for the adult trees: number of different alleles (A_n), allelic richness (A_r), observed heterozygosity (H_o), Nei's unbiased expected heterozygosity (H_e) and Wright's inbreeding coefficient (F_{IS}), using GenAlEx (version 6.2; Peakall & Smouse 2006). When calculating allelic richness, the rarefaction approach described by El Mousadik and Petit (1996) was used to account for differences in sample size. The percentage of rare alleles for the adults per study plot was calculated by dividing the number of low-frequency (< 0.05) alleles by the total number of alleles present in a stand.

Second, we verified whether the adult trees that covered the seed traps with their canopies were seed parents by performing a simple exclusion analysis based on the multilocus genotypes of seedlings and mother trees (Nakanishi et al. 2005). Seedlings with genotypes incompatible with those of their putative mothers were eliminated from the seed families that were used in further analyses. To investigate the effect of stand characteristics on pollen competition between distant and nearby trees, we estimated the proportion of pollen that originated from fathers within the study plot in each forest stand. Therefore, we performed a maximum likelihood paternity analysis using CERVUS 3.0.3. (Marshall et al. 1998) to assign paternity to the grown seedlings. The used set of microsatellite markers yielded high exclusion probabilities for paternal assignment (> 0.995). The most likely father trees were detected using LOD (log-likelihood ratio) scores (Marshall et al. 1998), which were based on the following simulation parameters: 10,000 simulated offspring, 0.01 (default settings) as the proportion of mistyped loci, and the number of candidate fathers ranging between 32 and 704 individuals (all mature trees in the studied forest stands). Father-offspring matches were conducted using the CERVUS 80% threshold level, and were verified by a straightforward exclusion analysis based on the genotypes of seedlings and adult trees (Nakanishi et al. 2005). When the threshold LOD-score for paternity was exceeded, the adult tree with the highest LOD-score, was designated as the father tree of a seedling. Based on the Euclidean distance

between pollen donors and maternal trees (distance from the stem), we calculated the average realized pollination distances within the study plots.

Since forest fragmentation may lower the opportunity to trees for outcrossing and potentially result in increased inbreeding, we characterized in each study plot a range of mating system parameters. This was done by using the mixed and correlated mating model implemented in the software package MLTR version 3.2 (Ritland 2002). MLTR was only applied to seed families composed of more than 5 seedlings per mother tree. Based on maximum likelihood procedures, we estimated single-locus (t_s) and multilocus (t_m) outcrossing rates, biparental inbreeding (t_m - t_s), and multilocus correlated paternity (r_p). Standard errors and confidence intervals (95%) were estimated for the mating system parameters using 1000 bootstrap replicates, re-sampling families within forest stands. Deviations from random mating through biparental inbreeding and correlated paternity may possibly increase the relatedness of the seedlings within seed families. This was examined by using the results of the mating system analysis for the calculation of the average coancestry coefficient within families (Θ_{xy}), which was estimated following Sousa et al. (2005): $\Theta_{xy} = 0.125(1+F_p)(1-r_p)$, with F_p the inbreeding coefficient in the parental generation. Additionally, we investigated the genetic structure within progeny by estimating the variance effective size within seed families: $N_{e(v)} = 0.5/\Theta_{xy}$ (Cockerham 1969). In an idealized panmictic population the maximum value of $N_{e(v)}$ within families corresponds to four unrelated individuals. However, deviations from random mating may reduce $N_{e(v)}$. Also the microsatellite data of the 35 adult trees within the 8 study plots was examined for possible significant spatial autocorrelation, by calculating the Nason's kinship coefficient F_{ij} (Loiselle et al. 1995) and their confidence intervals (95%; 10,000 permutations) using SPAGeDi 1.3 (Hardy & Vekemans 2002) (Appendix 4.2).

The diversity of the male gametes that contribute to the offspring of each mother tree may also shape the differentiation of the pollen gene pool among seed trees. To quantify the divergence in sampled pollen pools among the mother trees, the pollen pool differentiation (Φ_{FT}) among maternal plants was estimated with the

TWOGENER method (Smouse et al. 2001) as implemented in the software package POLDISP 1.0c (Robledo-Arnuncio et al. 2007). This analysis uses an AMOVA (analysis of molecular variance) approach, and is based on the genotypes of the seed families and their seed trees combined with spatial data of the mother trees (Excoffier et al. 1992). Confidence intervals (95%) around Φ_{FT} estimates were computed using 10,000 permutations among progeny. Finally, we also calculated the total genetic differentiation between families within the eight studied forest stands. This was done based on Nei's unbiased genetic distance (*D*) between seed families using GENALEX (version 6.2, Peakall & Smouse 2006).

4.3.5 Correlations of pollen flow and mating system parameters with forest stand characteristics

A Spearman's rank correlation coefficient (r_s) was used to investigate the relationships between stand characteristics (population size, tree density and isolation) and measures of pollen flow and mating system parameters. We applied a nonparametric test, since the number of data points was limited. The stand characteristics were not significantly correlated to each other ($r_{s\,(\text{size vs. isolation})} = 0$; $r_{s\,(\text{size vs. density})} = 0.48$ and $r_{s\,(\text{density vs. isolation})} = -0.59$, all $p > 0.05$). The effect of the landscape matrix (forest vs. agricultural land) on the estimated pollen flow and mating system parameters was examined using a Mann–Whitney U test. The forest stands that were located within agriculture land were characterized by a higher degree of isolation and tended to have lower tree densities. All statistical analyses were performed using SPSS (SPSS 20.0; SPSS Inc., Chicago, IL).

4.4 Results

A high percentage (> 90%) of seedlings was successfully grown from the acorns collected in the seed traps. In total, we sampled leaves of 281 adult trees and 396 grown seedlings. The nine microsatellites were highly variable, with the number of alleles per locus ranging from 11 (QpZAG9) to 36 (QpZAG104), with an average of 22.9 (± 2.8) (means are followed by SE in parentheses). Most stands showed high levels of genetic diversity in the adult cohort ($A_r > 11.3$; $H_o > 0.772$ and $H_e > 0.803$). In

the stand Hoge Vijvers, however, lower estimates for A_r = 8.5 (± 1.0); H_o = 0.728 (± 0.037) and H_e = 0.763 (± 0.034) were found (Table 4.2). The vast majority of the 396 grown seedlings (96%) showed genotypes that were consistent with the genotypes of their putative mother trees. In total, we detected 38 seed families that were composed of more than 5 seedlings per mother tree, and which were used for the estimation of gene flow and mating system parameters in the 8 stands (Table 4.1).

Table 4.2: Estimates of genetic diversity measures (± standard errors) for adults from eight *Q. robur* populations based on 9 microsatellite loci.

Forest stand	A_n	A_r^*	Rare alleles (%)	H_o	H_e	F_{IS}
Keffers	14.1 (1.5)	12.1 (1.2)	62.2 (4.9)	0.772 (.036)	0.825 (.028)	0.064 (.029)
Vos	14.0 (1.6)	12.0 (1.3)	58.8 (6.2)	0.819 (.041)	0.823 (.033)	0.005 (.033)
Hornebos	13.0 (1.6)	11.4 (1.3)	51.5 (5.5)	0.793 (.039)	0.818 (.028)	0.031 (.039)
Chartreuse	12.3 (1.6)	11.3 (1.4)	50.1 (3.7)	0.808 (.025)	0.818 (.033)	0.012 (.020)
Overheide	13.3 (1.6)	11.4 (1.3)	51.3 (5.4)	0.775 (.028)	0.803 (.024)	0.035 (.024)
Hoge Vijvers	9.3 (1.1)	8.5 (1.0)	41.1 (7.1)	0.728 (.037)	0.763 (.034)	0.046 (.028)
Meikensbos	14.0 (1.8)	12.2 (1.5)	60.4 (5.8)	0.800 (.041)	0.805 (.034)	0.006 (.027)
Egemse Veldekens	12.8 (1.4)	11.3 (1.2)	55.2 (5.3)	0.775 (.039)	0.803 (.029)	0.035 (.033)
Mean	12.9 (0.5)	11.4 (0.4)	53.8 (2.4)	0.784 (.010)	0.807 (.007)	0.029 (.007)

A_n, *number of different alleles*; A_r, *allelic richness;* H_o, *observed heterozygosity;* H_e, *expected heterozygosity;* F_{IS}, *inbreeding coefficient;*allelic richness based on a minimum sample size of 23 individuals*

When we compared the proportion of pollen that originated from nearby trees (within the study plot) with that of more distant trees, we found extensive pollen flow (0.50 - 0.77) from outside the study plot in most forest stands. Only in the stands Chartreuse and Hornebos a larger proportion of seeds originated from fathers within the study plot (Table 4.3). Stand characteristics significantly influenced the distribution of the pollen pools, as the proportion of pollen donors found within the study plots was significantly related to the size of the stands (r_s = -0.74, p = 0.037, Figure 4.1A) and tree density (r_s = -0.74, p = 0.037, Figure 4.1C). Besides decreased out-

Table 4.3. The results of the maximum likelihood paternity analysis conducted with CERVUS 3.0.3.

Forest stand	N_{seeds}	$N_{local\ fathers}$	EP_p	Proportion local fathers	Proportion of the seed cohort with different father trees	Mean pollen dispersal distance (m) [a]
Keffers	45	13	0.998	0.289	0.86 (0.07)	18.8 (2.9)
Vos	45	14	0.999	0.311	0.66 (0.22)	13.4 (1.9)
Hornebos	46	35	0.999	0.761	0.63 (0.14)	32.9 (2.5)
Chartreuse	49	29	0.999	0.592	0.64 (0.09)	20.0 (2.8)
Overheide	46	15	0.998	0.326	0.75 (0.19)	17.2 (2.1)
Hoge Vijvers	48	11	0.995	0.229	0.73 (0.18)	17.0 (2.6)
Meikensbos	50	25	0.998	0.500	0.74 (0.12)	22.4 (3.1)
Egemse Veldekens	48	21	0.998	0.438	0.89 (0.07)	47.5 (8.5)

N_{seeds}, *the number of seeds examined in the paternity analysis;* $N_{local\ fathers}$, *the number of seeds assigned to a father tree from within the study plot;* EP_P, *exclusion probabilities for paternal assignment; proportion local fathers,* $N_{local\ fathers}$ / N_{seeds}.

[a] *Within the study plot*

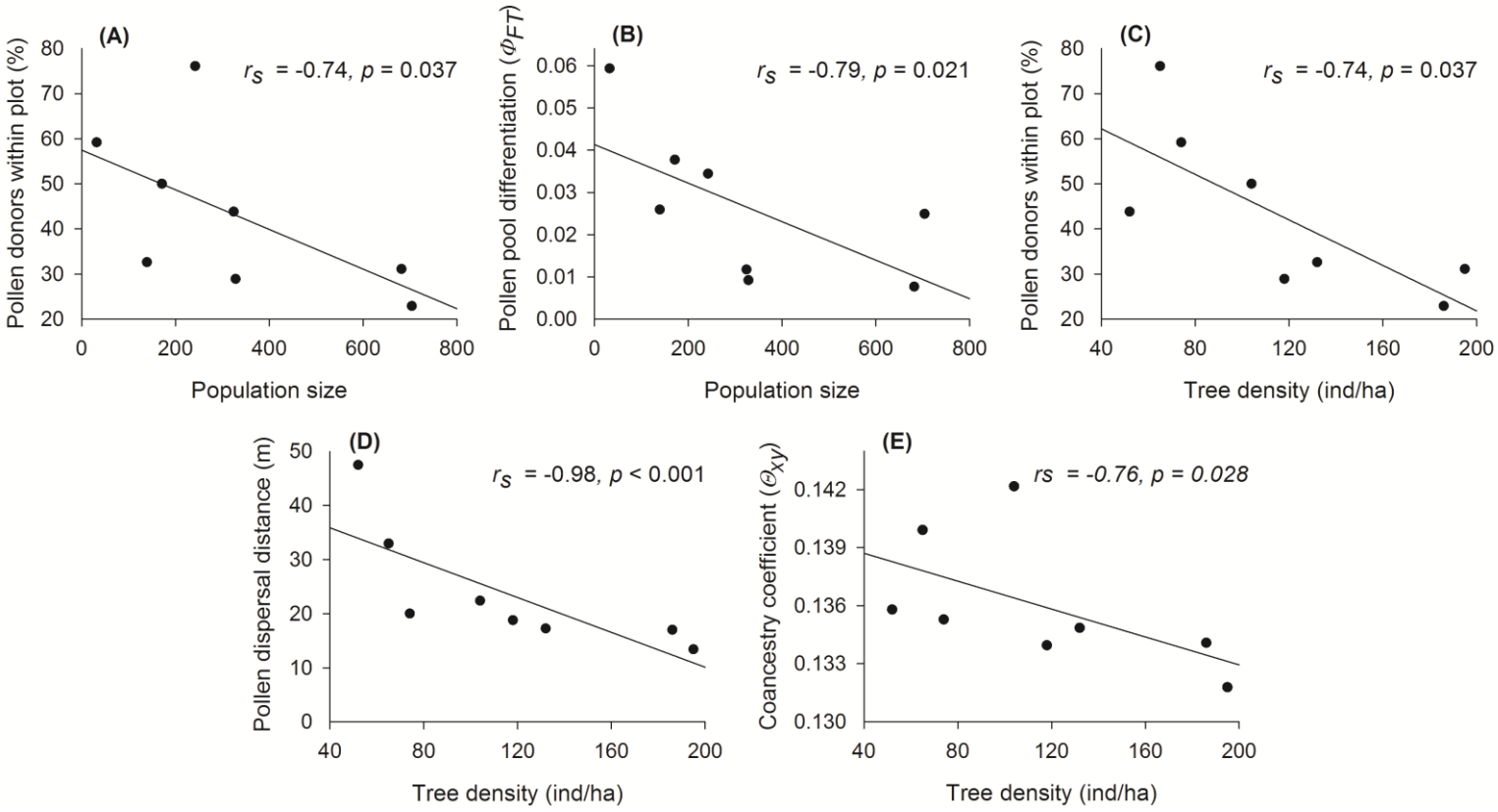


Figure 4.1. Spearman rank correlations (r_s) between stand characteristics and mating system and pollen dispersal parameters for eight forest stands of *Quercus robur*. (A) Percentage pollen donors from within the study plot (based on paternity assignment, CERVUS) vs. population size, (B) pollen pool differentiation (Φ_{FT}, based on TWOGENER method) vs. population size, (C) percentage pollen donors from within the study plot vs. tree density, (D) mean pollen dispersal distance within study plots (based on paternity assignment) vs. tree density, (E) coancestry coefficient within families (Θ_{xy}, based on estimates obtained with MLTR) vs. tree density.

of-plot pollen flow, low density forest stands also showed significantly higher genetically realized pollen flow distances within the study plot (r_s = -0.98, p < 0.001, Figure 4.1D). Furthermore, these effective pollination distances were significantly higher in forest stands surrounded by agricultural land (Z = -2.24, p = 0.036).

Reductions in stand size or conspecific density did not alter the opportunity for outcrossing in the studied plots, as all forest stands showed high multilocus outcrossing rates (t_m > 0.998), indicating that selfing was almost completely lacking (s ≤ 0.002, Table 4.4). Moreover, the level of biparental inbreeding (t_m - t_s) was also low (0.025 - 0.118), although values were significantly different from zero in all plots. The average biparental inbreeding per study plot was significantly and positively related to the estimated mean genetic differentiation (Nei's D) between seed families within study plots (r_s = 0.83, p = 0.010, Figure 4.2A). Next to biparental inbreeding, most study plots showed a significant (p < 0.05) presence of full sibs within the seed families, only in the stands Keffers and Hoge Vijvers the 95% confidence intervals of correlated paternity (r_p) included zero (Table 4.4). The above deviations from random mating also increased the relatedness of the seedlings within seed families. The average coancestry coefficient (Θ_{xy}) was higher (0.132 - 0.142) than the value expected in half-sib families (Θ_{xy} = 0.125), whereas the variance effective size within seed families ($N_{e(v)}$) was lower than 4 (3.52 - 3.80), indicating the presence of an increased genetic structure within the progeny cohort. Contrary to the grown seedlings, no significant genetic structure within the adult generation was found in the 8 study plots, as the mean kinship coefficient F_{ij} fell within the 95% confidence interval for all distance classes (Appendix 4.2). Although the range in coancestry coefficients was restricted, stand characteristics influenced the relatedness of the seedlings, as Θ_{xy} was significantly negatively related to tree density (r_s = -0.76, p = 0.028, Figure 4.1E) and increased with increasing pollen flow distances within the study plots (r_s = 0.83, p = 0.010). Furthermore, significantly higher estimates of Θ_{xy} were found in forest stands surrounded by a matrix of agricultural land (Z = -2.26, p = 0.036). Mating system parameters were also affected by the diversity of the male gametes that contributed to the pollen pool. Both r_p and Θ_{xy} estimates strongly decreased with increasing

pollen flow from outside the study plot (r_s = -0.81, p < 0.015, Figure 4.2B and r_s = -0.79, p < 0.021 respectively).

Finally, the estimates of the TWOGENER analysis showed significant pollen pool differentiation among maternal plants (Φ_{FT}) in most of the forest stands (95% CI excludes zero) and were significantly negatively correlated to stand size (r_s = -0.79, p = 0.021, Figure 4.1B). Pollen pool differentiation among mother trees was also affected by the relatedness of the seedlings, as we found that Φ_{FT} estimates increased with increasing values of Θ_{xy} (r_s = 0.74, p = 0.037, Figure 4.2C).

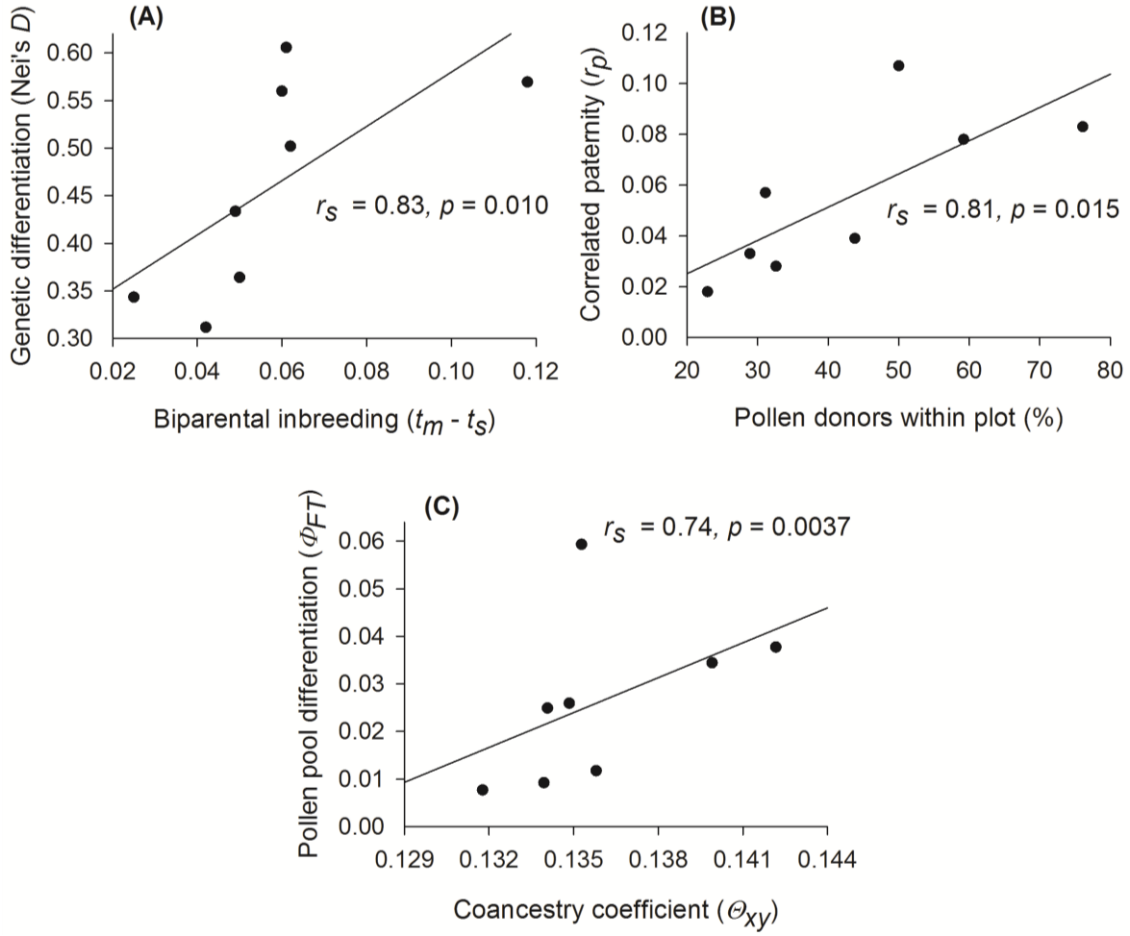


Figure 4.2. Spearman rank correlations (r_s) between mating system and pollen dispersal parameters for eight forest stands of *Quercus robur*. (A) Unbiased genetic differentiation between seed families (Nei's *D*) vs. biparental inbreeding (t_m - t_s), (B) correlated paternity (r_p) vs. the percentage pollen donors from within the study plot, (C) pollen pool differentiation (Φ_{FT}) vs. the coancestry coefficient within families (Θ_{xy}).

Table 4.4. Mating system estimates and pollen pool differentiation (with standard errors in parentheses) for the eight studied *Q. robur* stands.

Forest stand	t_m	t_s	$t_m - t_s$	r_p	$1/r_p$	Θ_{xy}	$N_{e(v)}$	Φ_{FT}
Keffers	0.998 (0.000)	0.949 (0.009)*	0.049 (0.009)*	0.033 (0.021)	30.3	0.134	3.73	0.009 (0.007)
Vos	0.998 (0.001)	0.948 (0.010)*	0.050 (0.009)*	0.057 (0.020)*	17.5	0.132	3.80	0.008 (0.007)
Hornebos	0.998 (0.000)	0.973 (0.006)*	0.025 (0.006)*	0.083 (0.017)*	12.1	0.140	3.57	0.034 (0.008)*
Chartreuse	0.998 (0.000)	0.937 (0.012)*	0.061 (0.012)*	0.078 (0.026)*	12.8	0.135	3.70	0.059 (0.007)*
Overheide	0.999 (0.001)	0.936 (0.008)*	0.062 (0.007)*	0.028 (0.005)*	35.7	0.135	3.71	0.026 (0.005)*
Hoge Vijvers	0.999 (0.000)	0.881 (0.010)*	0.118 (0.010)*	0.018 (0.011)	55.6	0.134	3.73	0.025 (0.005)*
Meikensbos	0.998 (0.000)	0.956 (0.008)*	0.042 (0.008)*	0.107 (0.042)*	9.4	0.142	3.52	0.038 (0.006)*
Egemse Veldekens	0.999 (0.000)	0.939 (0.011)*	0.060 (0.011)*	0.039 (0.008)*	25.6	0.136	3.68	0.012 (0.006)*

t_m, *Multilocus outcrossing rate;* t_s, *singlelocus outcrossing rate;* $t_m - t_s$, *biparental inbreeding;* r_p, *correlated paternity estimated with Ritland's mixed mating model (2002);* $1/r_p$, *effective number of pollen donors;* Θ_{xy}, *coancestry coefficient within families;* $N_{e(v)}$, *variance effective population size within families;* Φ_{FT}, *Pollen pool differentiation among maternal plants estimated with the TWOGENER method (Smouse et al. 2001).*

**Significantly less than 1 for* t_m *and* t_s, *and significantly greater than zero for* $t_m - t_s$, r_p *and* Φ_{FT} ($p < 0.05$).

4.5 Discussion

4.5.1 Rates of pollen dispersal into and within the study plots

The high proportions of out-of-plot pollen-mediated gene flow (0.24 - 0.77) that were found in our study, are consistent with those detected in previous gene flow studies in *Quercus* species (0.35 - 0.70: Streiff et al. 1999; Nakanishi et al. 2005; Valbuena-Carabana et al. 2005; Chybicki & Burczyk 2010; Vranckx et al. 2014). Similar to our study, the plots in these studies were part of larger forest stands and were thus characterized by a relatively low level of geographic isolation from neighboring oak trees (< 1000 m). It can be expected that when the scale of isolation between neighboring and distant trees exceeds the scale of pollen dispersal, pollen exchange will be much more restricted, even in wind-pollinated tree species (Ellstrand 1992; Sork & Smouse 2006). This was demonstrated by Schuster and Mitton (2000) and Bittencourt and Sebbenn (2007), who obtained lower rates of out-of-plot pollen flow (0.065 and 0.1 respectively) in stronger isolated (> 2000 m to the nearest adult tree) study plots of *Pinus flexilis* and *Araucaria angustifolia*. Since our study plots (35 genotyped adult trees) were surrounded by a large proportion of ungenotyped father trees, one would expect high rates of pollen flow into all of our study plots. However, the proportion of seeds that originated from fathers from outside our study plots varied strongly between forest stands. For example out-of-plot pollen flow was three times higher in the forest stand Hoge Vijver (0.77) compared to the plot located in Hornebos (0.24). Contrary to the findings of Robledo-Arnuncio et al. (2004), who observed increased pollen flow rates after conspecific tree density decreased, we found significant positive correlations between out-of-plot pollen flow and the number and density of adult trees within the forest stands. Reduced adult numbers may decrease the proportion of ungenotyped adult trees, relative to the total forest stand, whereas low conspecific density will increase the pollination distances between the 35 genotyped adults and the ungenotyped trees from outside the study plot (Smouse & Sork 2004; Bianchi & Cunningham 2012; Breed et al. 2012). As a result, a larger proportion of pollen in the local pollen pool may originate from adult trees within the study plot.

Based on the paternity analysis performed in Cervus, we also demonstrated that the Euclidean distances between pollen donors and mother trees were strongly negatively correlated with the tree density of the forest stands, and were significantly higher in forest stands surrounded by agricultural land. It has been shown before in *Fraxinus excelsior*, *Q. robur* and *Q. petraea* that low conspecific tree density increased the average distance between mates (Robledo-Arnuncio et al. 2004; Bacles & Ennos 2008; Wang et al. 2010). The most likely explanation for this observation is that pollen competition between distant and nearby trees decreases when there are fewer nearby trees available as a pollen donor, through which the average realized pollination distance will be greater. Furthermore, in more open landscapes, airborne pollen movement will be facilitated, as winds in open landscapes are typically stronger than winds in a canopy closed forest matrix (Nathan et al. 2002; Bacles et al. 2005).

4.5.2 Mating patterns and local pollen diversity

Our results suggest that not only population size, but also tree density has a major impact on local pollen flow patterns, and may ultimately influence mating patterns and local pollen diversity in small scale forestry systems of *Q. robur* (Stacy et al. 1996; Kamm et al. 2009). Although high outcrossing rates ($t_m > 0.998$) were observed in all stands, the estimates of both the average coancestry coefficient ($\Theta_{xy} > 0.125$) and variance effective size within families ($N_{e(v)} < 4$) suggested that seeds within seed families were stronger related than what was expected under panmixia (Sousa et al. 2005; Bittencourt & Sebenn 2008). Such deviations from random mating can be attributed to several factors such as selfing, correlated paternity and mating among relatives (Robledo-Arnuncio et al. 2004; Bittencourt & Sebenn 2008). Since oak species possess a nearly complete gametophytic self-incompatibility system (Ducousso et al. 1993; Steinhoff 1993), only negligible selfing rates could be detected ($s = 0.1 - 0.2\%$). In contrast, low but significant levels of correlated paternity and biparental inbreeding were obtained, which may both have contributed to the observed genetic structure within the studied seed families. The non-significant spatial genetic structure that was observed in the adult generation can be attributed to several factors, such as: tree

planting in the past, the restricted spatial scale of the adult plots (only 32 - 35 trees) and the limited number of samples used in the SGS analyses.

Although the range in coancestry coefficients was restricted, the relatedness of the seeds within families decreased, with increasing tree density. Reduced local tree density may alter mate availability, such that the number of nearby pollen sources surrounding a mother tree decreases and the pollination distance between mates increases (Sork et al. 2002; Eckert et al. 2010; Breed et al 2013b). However, this larger average effective pollination distance did not totally compensate for the reduction in conspecific tree density, through which the diversity of the pollen pool contributing to each seed tree was lower than under high tree density, which ultimately resulted in slightly more related progeny (Robledo-Arnuncio et al. 2004; Sork & Smouse 2006). Next to tree density, the landscape matrix significantly influenced the relatedness of the seeds within seed families. Although we found some evidence for higher pollen flow distances in more open landscapes, higher coancestry coefficients for the seedling cohort were found, indicating less diverse pollen pools compared to forest stands that were located in a matrix of other forests. This is in contrast with what was expected based on the study of Bacles and Ennos (2008), who found higher pollen pool diversities in forest remnants that were located in open landscapes. The effects of the landscape matrix could, however, be confounded by stand isolation and tree density in our study, as the forest stands that were surrounded by a matrix of agricultural land, were much more isolated from the nearest *Q. robur* stand than the stands located in a forest matrix and tended to have lower tree densities. This could have lowered the number of pollen sources contributing to the local pollen pool, both as a result of increased pollination distances between mates and through reduced gene inflow from outside the forest stands when isolation between stands became too high (Sork & Smouse 2006). Reduced mate availability may ultimately have led to less diverse local pollen pools and stronger biparental inbreeding, through which the proportion of outcrossed half- and full-sibs in the progeny cohort increases (Lowe et al. 2005; Sork et al. 2002). High levels of pollen exchange may possibly mitigate the negative effects of small and low density forest stands on mating patterns, through enlarging the diversity of pollen sources within the local pollen pool (Sork & Smouse

2006). Such positive effect of strong pollen flow has been demonstrated in previous studies in wind-pollinated tree species (O'Connell et al. 2006; Bittencourt & Sebenn 2007; Wang et al. 2010), and was also confirmed in our study, as we found a significant negative correlation between pollen flow from outside the study plots and the estimates of both the correlated paternity and coancestry coefficient.

Next to the slightly higher coancestry coefficients, the occurrence of correlated paternity and biparental inbreeding may also produce pollen pool differentiation among seed parents (Austerlitz & Smouse 2001; Hardy et al. 2004; Bittencourt & Sebbenn 2007). Our study supports this, as the measures that were used to quantify genetic differentiation between pollen pools and seed families (Φ_{FT} and Nei's D respectively) showed positive, significant relationships with biparental inbreeding and correlated paternity. The estimates of pollen pool heterogeneity obtained with the TWOGENER approach (Φ_{FT} = 0.008 - 0.059) were significant in 6 out of 8 stands, and were consistent with Φ_{FT}-values reported in previous oak studies (Φ_{FT} = 0.011 - 0.087) (Smouse et al. 2001; Nakanishi et al. 2005; Fernández-Manjarrés et al. 2006; Pakkad et al. 2008). Furthermore, Φ_{FT}-estimates significantly increased with decreasing population size, suggesting less diverse local pollen pools, and consequently, an increased likelihood of consanguineous matings in small forest stands (Robledo-Arnuncio et al. 2004). In general, any factor (tree density, stand size, isolation, etc.) that may disrupt mating patterns and genetic structure within forest stands, will increase the differentiation among maternal pollen pools (Austerlitz & Smouse 2001; Gram & Sork 2001).

4.5.3 Implications for current and future forest management

The presence of significant levels of correlated mating and reduced local pollen diversity may have implications for forest management aiming at maximizing genetic diversity in fragmented *Q. robur* stands. First, compared to random mating conditions, our results suggest that a larger number of seed trees have to contribute to the offspring cohort to retain minimum effective population sizes within forest stands (Bittencourt & Sebbenn 2008). This is especially important when forest reproductive material used for artificial regeneration is harvested in one of these

forest stands. Preferably seeds should be harvested in one or several large, continuous and higher-density forest stands, to avoid the collection of inbred seed material (Breed et al. 2013a). Second, our data showed significant relationships between stand characteristics (population size, tree density and landscape matrix) and several mating and pollen flow parameters, emphasizing the role of forest management in shaping mating patterns and pollen-mediated gene flow within *Q. robur* stands. Removal of adult trees and decreasing tree density directly increased the genetic structure within the studied offspring cohort. This may not only affect future tree fitness through increasing the likelihood of consanguineous matings (among half and full-sibs) in subsequent generations (Young & Brown 1999), but the significant levels of pollen pool differentiation and reduced local pollen diversity may directly reduce pollen competition within the paternal mating pool, through which the proportion of recessive deleterious alleles increases in the progeny cohort (Breed et al. 2012).

Our results should be interpreted with some caution, as the studied seedlings were grown under controlled greenhouse conditions, whereas natural selection in the field may possibly shape patterns of relatedness across generations. A gradual decrease of less fit homozygotes from the seedling cohort to the adult generation will potentially reduce the number of individuals that originated from biparental inbreeding in the adult generation. Furthermore, we collected seeds from one masting year only, whereas adult trees may reproduce during many decades. Nonetheless, light-demanding tree species such as *Q. robur* are characterized by a narrow recruitment window, restricted in time (mast years) and space (light requirement) and therefore the number of reproduction events contributing to the future adult generation are limited (Vera et al. 2006; Bary-Lenger & Nebout 1993). Finally, part of the shortcomings in this study resulted from the limited number of populations and individuals that were examined. Consequently, some of the stand characteristics (isolation and density) were confounded with the landscape matrix. To disentangle these confounding effects and to allow rigorous statistical testing of the effects of stand characteristics on mating and pollen flow patterns, more forest stands differing in tree density, isolation and matrix type were necessary. The

analyses of multiple forest stands in our study also had a price: sample sizes in all stands were restricted. The examination of more seed trees, adults and seedlings per site would have made the estimations of geneflow and mating system parameters much more robust.

Since small and low density forest stands are common in the extremely fragmented forest landscapes of Northern Belgium and other parts of Europe (Vandekerkhove 2013), high levels of pollen flow within and between forest fragments should be maintained to counteract the negative genetic processes associated with forest fragmentation. However, increasing the genetic connectivity of natural regenerated forest stands is not always realistic in highly fragmented forest landscapes, through which more customized management practices are needed to maintain or increase N_e. Selecting the best regeneration method (natural or artificial regeneration, or both) should be determined by its likelihood to maintain a high genetic diversity on the long term, and will consequently depend on the characteristics of the forest stand.

Chapter 5.

The effect of drought stress on heterozygosity-fitness correlations in pedunculate oak (*Quercus robur* L.)

Adapted from:

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Janssens P, Gielen BAS, Muys B, Honnay O. 2014. The effect of drought stress on heterozygosity-fitness correlations in pedunculate oak (*Quercus robur*). *Annals of Botany* 113(6): 1057-1069.

5.1 Abstract

The interaction between forest fragmentation and predicted climate change may pose a serious threat to tree populations. In small and spatially isolated forest fragments, increased homozygosity may directly affect individual tree fitness through the expression of deleterious alleles. Climate change-induced drought stress may exacerbate these detrimental genetic consequences of forest fragmentation, as the response to low levels of individual heterozygosity is generally thought to be stronger under environmental stress than under optimal conditions. To test this hypothesis, a greenhouse experiment was performed in which various transpiration and growth traits of 6-month-old seedlings of *Quercus robur* differing in multilocus heterozygosity (MLH) were recorded for 3 months under a well-watered and a drought stress treatment. Heterozygosity-fitness correlations (HFC) were examined by correlating the recorded traits of individual seedlings to their MLH and by studying their response to drought stress. Weak, but significant, effects of MLH on several fitness traits were obtained, which were stronger for transpiration variables than for the recorded growth traits. High atmospheric stress (measured as vapour pressure deficit) influenced the strength of the HFCs of the transpiration variables, whereas only a limited effect of the irrigation treatment on the HFCs was observed. Under ongoing climate change, increased atmospheric stress in the future may strengthen the negative responses of trees to low MLH. This indicates the necessity to maximize individual multilocus heterozygosity in forest tree breeding programmes.

5.2 Introduction

Forests are essential to life on earth as they provide a multitude of ecosystem services, including climate mitigation, water regulation and biomass production (Lindenmayer & Franklin 2002; Thompson et al. 2009). Over recent decades the functioning and sustainability of forests have been increasingly challenged by various anthropogenic threats (Simberloff 1999; Millennium Ecosystem Assessment 2005). One of the most important threats is climate change (Noss 2001; Lindner et al. 2010). For Europe, climate projections predict increasing temperatures and irregular precipitation patterns during summer, which will increase the number and intensity of drought events (Stocker et al. 2013). During such events, soil water shortage can be expected to induce the closure of stomata, which may directly damage leaf tissue of trees through the inhibition of leaf cooling (Bréda et al. 2006). At more severe levels of drought stress, water transfer within the xylem may be irreversibly disrupted hrough vessel embolism (cavitation), which may result in losses of roots or twigs. Next to cavitation, tree mortality after long-term drought may also be caused by carbon starvation. This process induces the depletion of carbon resources during drought stress, as stomatal closure will lower carbon assimilation such that it is insufficient to supply the required amounts of carbon (McDowell et al. 2008). Drought-induced physiological disorders will not only affect biomass production in the short term, but may also increase the susceptibility of trees to secondary stresses such as frost, fungal infections and insect attacks, which may affect tree health and eventually lead to tree death (Bréda et al. 2006; Lindner et al. 2010).

Next to climate change, the loss and fragmentation of forests through land-use changes poses a second major worldwide threat to forest sustainability (Hamrick 2004; Millennium Ecosystem Assessment 2005). Forest fragmentation results in small and spatially isolated forest fragments in which increased random genetic drift and inbreeding may erode genetic diversity of the tree populations (Jump & Peñuelas 2006; Vranckx et al. 2012). Genetic diversity is crucial, however, for the maintenance of vital and productive forests (Jump et al. 2009), since population genetic variation provides the raw material for evolution, allowing adaptation of forest trees to

environmental changes (Willi et al. 2006). Moreover, increased homozygosity resulting from inbreeding may directly affect individual tree fitness, through the expression of deleterious alleles that influence morphological, physiological and life-history traits. The relationships between heterozygosity and fitness traits are generally known as heterozygosity-fitness correlations (HFCs), and are more likely to occur in small, non-random mating populations that typically occur in fragmented habitats (David 1998; Hansson & Westerberg 2002; Szulkin et al. 2010). Quantifying HFCs may give insight into the effects of inbreeding on individual tree performance (Hedrick & Kalinowski 2000; Reed & Frankham 2003) and is also relevant to forest management and forest tree breeding programmes designed to maximize biomass production (Aravanopoulos & Zsuffa 1998; Alig et al. 2003).

The detrimental genetic consequences of forest fragmentation may be exacerbated by climate change-induced drought stress because the response of tree species to low levels of heterozygosity is generally thought to be more pronounced under environmental stress than under optimal conditions (Armbruster & Reed 2005). However, the effects of environmental stress on HFCs are not always clear (Keller & Waller 2002), and beyond a certain level of stress HFCs may become less apparent (Audo & Diehl 1995; David 1998). Whereas the relationships between heterozygosity and fitness traits have been examined frequently in conifers (e.g. Mitton et al. 1981; Ledig et al. 1983; Bush et al. 1987; Savolainen & Hedrick 1995), similar studies are rare for broadleaved species (Aravanopoulos & Zsuffa 1998). Furthermore, we are not aware of any study that has focused on the potentially detrimental interaction between climate change and habitat fragmentation by studying the effects of drought stress on HFCs.

Here we investigated HFCs and their response to drought stress in the economically important broadleaved tree species pedunculate oak (*Quercus robur*). We selected different transpiration variables and various growth traits as fitness variables (Van Hees 1997; David 1998; Bréda et al. 2006). First, growth is an important fitness component, especially in indeterminate growers such as trees, which are characterized by size-dependent fecundity (David 1998). Rapid early

growth and strong biomass production will increase the competitive ability of seedlings, through which they may outcompete neighbouring seedlings when competition for light and resources is strong (King 1981; Bush et al. 1987; Scotti-Saintagne et al. 2004). Furthermore, since the crown size of many tree species is strongly correlated with stem diameter (Hemery et al. 2005), larger oak trees may also have greater crown areas for flowering and acorn production (Greenberg 2000). Second, transpiration variables, such as stomatal conductance, water potential and the water content of seedlings, may give an indication of the water status of a plant and may influence the physiological processes that determine carbon fixation and growth (Bréda et al. 2006). Low soil water content and high atmospheric evaporative demand may decrease the leaf water potential of oak seedlings and induce stomatal closure (Fort et al. 1997; Cavender-Bares & Bazzaz 2000). This may reduce the rate of stomatal conductance, limiting water fluxes at the cost of reduced photosynthesis and biomass production (Bréda et al. 2006). Furthermore, although transpiration efficiency (ratio of biomass production to transpiration) generally increases during drought stress, Donovan and Ehleringer (1991) and Cavender-Bares and Bazzaz (2000) have suggested that this increase in transpiration efficiency may be lower during seedling establishment, when it is accompanied by increased seedling growth.

HFCs are likely to emerge in Q. *robur* since forest stands of this tree species are small in many parts of Western Europe due to past deforestation and fragmented forest ownership (Wiersum et al. 2005). Moreover, recent research has revealed variation in individual multilocus heterozygosity within small oak stands in northern Belgium, despite high heterozygosity levels at the population scale (Vranckx et al. 2014). Therefore, it can be hypothesized that:

- This within-stand variation in individual multilocus heterozygosity may be correlated to the variation in transpiration and growth traits of the seedlings
- These HFCs are stronger under stress conditions.

To test these hypotheses, we quantified multilocus heterozygosity based on nine neutral microsatellite loci in 150 seedlings originating from three populations (50

seedlings per population). A greenhouse experiment was performed in which seedlings of *Q. robur* that differed in multilocus heterozygosity were grown under standardized environmental conditions. Various transpiration and growth traits of 6-month-old seedlings were recorded for 3 months under both a well-watered and a drought stress treatment.

5.3 Materials and Methods

5.3.1 Study species

Pedunculate oak (*Quercus robur* L.) is a keystone tree species of many European forest ecosystems, with a large natural range extending from southern Scandinavia to sub-Mediterranean Europe, and eastwards to the Ural Mountains (Bary-Lenger & Nebout 1993). This monoecious, wind-pollinated tree species occurs on a wide range of soils, and displays a medium degree of drought tolerance, which may be attributed to its deep rooting system and the maintenance of high rates of stomatal conductance during moderate levels of drought stress (Epron & Dreyer 1993). Compared with its closest congener *Quercus petraea*, *Q. robur* prefers to grow on neutral soils characterized by good water-holding capacity or soils with a permanent water table within reach of the root system (Bary-Lenger & Nebout 1993). Flower fertilization is followed by rapid development of acorns, which are dispersed during autumn by gravity, small rodents and birds. Although acorns can germinate and establish under a closed canopy, forest canopy openings are required for further seedling growth and development (Bary-Lenger & Nebout 1993).

5.3.2 Seed collection and experimental set-up

Acorns of *Q. robur* were collected in the autumn of 2011 in three small (< 4 ha) monospecific pedunculate oak stands in the centre of Flanders (Northern Belgium). These forest stands had been studied previously (Vranckx et al. 2014) and showed high heterozygosity values at the population level, which were consistent with what was found in other population genetic studies of *Q. robur* (Mariette et al. 2002; Hampe et al. 2010). The maximum distance between these stands was less than 15

km, and all were located in a matrix of forest stands composed of other tree species and/or agricultural land (Table 5.1). Pollen exchange among the three forest stands (Vos, Keffers and Chartreuse) was rather limited, as the minimum geographical distance between stands (~ 3400 m) was much greater than the average pollen dispersal distances (130 - 210 m) for the three studied stands, based on the neighbourhood model implemented in the program NM+ (Chybicki and Burczyk 2010). A circular plot containing 35 adult trees was established in the centre of each *Q. robur* stand, in which five seed traps (1.5 m^2 each) were randomly located. At the end of September, ten acorns from each seed trap were collected and stored at 5 °C for 4 weeks. Cold storage increased both the percentage and synchronization of seed germination (Manzanera et al. 1993). After cold storage, 50 *Q. robur* seeds per forest stand were weighed and sown in the centre of 3 L open-bottom pots filled with commercial soil (20% organic, pH ~ 6, electrical conductivity = 750 μS cm^{-1}, 14:16:18 N:P:K 1 kg m^{-3}). The pots were randomly placed in a greenhouse under controlled climatic conditions with a 12/12 h day/night light regime, and kept at field capacity using an automatic drip irrigation system. The irrigation water contained a Peters professional nutrient mixture (20:20:20 N:P:K, including trace elements; Everris International, Geldermalsen, the Netherlands). High germination percentages were obtained (> 95%), with most of the seeds germinating in week 4 after sowing.

Table 5.1. Characteristics of the three pedunculated oak stands were acorns were collected

forest stand	Latitude (*N*)	Longitude (*E*)	Area (ha)	Population size	Density (ind/m^2)	Plot size (ha)	Isolation (m)
Keffers	50°50'26''	4°42'00''	3.04	328	0.0118	0.49	400
Vos	50°49'27''	4°39'33''	3.97	682	0.0195	0.24	175
Chartreuse	50°54'45''	4°46'25''	0.43	32	0.0074	0.43	135

5.3.3 Microsatellite analyses

After 6 months growing (April 2012) under controlled climatic conditions all seedlings were genotyped. A single leaf was taken from each seedling and stored in silica prior to DNA extraction. Dried leaf samples (200 mg) were ground before DNA

extraction with a Nucleospin Plant II kit (Macherey-Nagel). We selected ten microsatellite loci that had been developed for *Q. petraea* (QpZAG9, QpZAG108, QpZAG46, QpZAG15, QpZAG110, QpZAG104; Steinkellner et al. 1997), *Quercus macrocarpa* (MSQ4, MSQ13, MSQ16; Dow et al. 1995; Dow & Ashley 1996) and *Q. robur* (QrZAG112; Kampfer et al. 1998). Polymerase chain reaction amplifications were carried out using a Multiplex PCR Master Mix kit (Qiagen) with the thermocycler programme of 15 min at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 50 °C and 45 s at 72 °C and final extension at 72 °C for 10 min. The amplified fragments were analysed with an ABI 3500 genetic analyser (Applied Biosystems, Foster City, CA, USA) and GeneMapper software (version 4.1). The microsatellite data were first analysed using the software Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004) to check for possible genotyping errors, including null alleles, stutter peaks and large allele drop-out. We observed a consistent high null allele frequency at one locus (MSQ16), which was removed from further analyses.

Neutrality of the microsatellites was first checked with the Ewens-Watterson homozygosity test of neutrality (Manly 1985) using Popgene version 1.32 (100,000 permutations; Yeh et al. 1999). This test compares observed allele frequencies with those expected under mutation-drift equilibrium, and is therefore useful for detecting deviations from neutrality due to selection or demographic changes. We found no significant departures from neutrality for any loci; however, the observed F (sum of squares of allele frequencies) for locus QpZAG104 was close to the lower limit of the 95% confidence interval (Appendix 5.1). Second, We also conducted a F_{st} outlier test implemented in Arlequin v.3.5. (Excoffier & Lischer 2010). This method evaluates the probability of a locus to be under selection, based on the relationship between F_{st} and expected heterozygosity (H_e). This relationship is then compared to a simulated neutral null distributions, which is obtained by simulating samples under a hierarchical island model using a coalescent approach (Excoffier et al. 2009). All the applied microsatellites showed non-significant *p*-values ($p > 0.05$) for the F_{st} outlier test, suggesting the neutrality of the loci (Appendix 5.2).

The following population genetic diversity measures were calculated for the studied seedlings: number of different alleles (A_n), allelic richness (A_r), observed heterozygosity (H_o), Nei's unbiased expected heterozygosity (H_e) and Wright's inbreeding coefficient (F_{IS}), using GenAlEx (version 6.2; Peakall & Smouse 2006). When calculating allelic richness, the rarefaction approach described by El Mousadik and Petit (1996) was used to account for differences in sample size. The percentage of rare alleles for the seedlings was calculated by dividing the number of low-frequency (< 0.05) alleles by the total number of alleles present in a stand.

Subsequently, three different metrics were calculated to detect HFCs in the genotyped oak seedlings. First, multilocus heterozygosity (MLH) was measured in each seedling as the percentage of microsatellite loci for which an individual was heterozygous, corrected for non-scored loci (Slate & Pemberton 2002; Chapman et al. 2009). A second microsatellite-based metric that has been proposed for the study of HFCs is mean d^2, which is the squared difference (in repeat units) between the two alleles at a locus, averaged over all the microsatellite loci for which an individual was examined (Coulson et al. 1998; Hedrick et al. 2001; Slate & Pemberton 2002). Mean d^2 was calculated as follows:

$$\bar{d}^2 = \frac{1}{N}\sum_{i=1}^{N}\left(n_{i1} - n_{i2}\right)^2,$$

where n_{i1} and n_{i2} are the lengths in repeat units of the two alleles at the ith locus and N is the number of scored loci examined in an individual. Finally, we also calculated the standardized mean d^2 (sMd^2), which limits the influence of highly polymorphic loci on the metric. Therefore, d^2 was standardized with its locus-specific variance (Pemberton et al. 1999). By studying the above genetic metrics, we actually investigated different ecological processes. MLH has been considered to better detect recent inbreeding events than d^2, whereas d^2 may also contain information about historical events in an individual's ancestry, such as the influence of population admixture (hybrid vigour, high d^2) (Coulson et al. 1998; Pemberton et al. 1999). To assess the relationship between the three tested genetic measures (MLH, d^2 and sMd^2), Spearman rank correlation coefficients (r_s) were calculated.

5.3.4 Treatment phase

After 6 months at field capacity, 100 of the 150 seedlings were exposed to the treatment phase (8 May 2012) and the 50 remaining seedlings were harvested for initial biomass estimation. The 100 seedlings subjected to the treatment phase were selected in such a way that individuals with high and low MLH values originated equally from all three forest stands. Subsequently, these seedlings were assigned to two irrigation treatments such that MLH levels and forest stands were uniformly represented within and between treatments. These drought stress regimes were established based on relative extractable soil water (REW):

$$\mathrm{REW} = \frac{\theta_V - \theta_{WP}}{\theta_{FC} - \theta_{WP}}$$

and measurements of the actual volumetric moisture content (θ_V) with a TRIME TDR FM3 sensor (IMKO, Ettlingen, Germany). The volumetric soil water contents at field capacity (θ_{FC}, suction pressure (pF) = 2.0) and at wilting point (θ_{WP}, pF = 4.2) were measured and calculated for the applied commercial soil (40.8 and 5.8%, respectively). The normal water treatment consisted of 50 seedlings subjected to a relative extractable soil water above 0.80 (θ_V = 33.8%), which greatly exceeds the general water deficit threshold for trees (REW = 0.30 - 0.40; Granier et al. 2007). To determine the effect of severe drought stress, the remaining seedlings were irrigated up to a relative extractable soil water content of 0.10 (θ_V = 9.3%). This value corresponds to the estimates (REW = 0.05 - 0.2) recorded by Bréda et al. (2006) in European forests during the severe summer drought of 2003. Target weights for watering were calculated for all seedlings based on relative extractable soil water and the relationship between pot weight and soil moisture content. This relationship was regularly checked (weeks 1, 4 and 10) and adjusted for changes in plant weight. Seedlings were watered three times a week up to their target weight and pots were weighed before and after irrigation. To prevent soil evaporation and percolation, the surface and bottom of the pots were covered with aluminium and plastic foil respectively. Five control pots without seedlings were weighed to correct for soil evaporation. During the entire experiment, air temperature (T_a), relative air humidity

(RH) and photosynthetically active radiation (PAR) were measured every 5 min. PAR was converted from Wm^{-2} to the more usual $\mu molm^{-2}s^{-1}$, based on the conversion factors of McCree (1972). Mean daytime values of T_a, PAR and vapour pressure deficit (VPD, calculated using T_a and RH) are given in Table 5.2. Pots were fully randomized every week to reduce position effects within the greenhouse. Sulphur vaporization and preventive spraying with acaricides (Floramite, Nissorun) were successfully applied for the control of powdery mildew (*Microsphaera alphitoides*) and red spider mite (*Tetranychus urticae*).

Table 5.2: Mean values and their standard errors (in parentheses) for air temperature (T_a), VPD and PAR during the treatment phase and during the measurements of stomatal conductance (g_s) and leaf water potential (ψ) in *Q. robur*

Time	Variable	T_a (°C)	VPD (kPa)	PAR ($\mu molm^{-2}s^{-1}$)
Treatment period				
Entire day		22.8 (0.1)	1.30 (0.017)	359.3 (29.9)
Morning		20.7 (0.1)	0.90 (0.019)	285.2 (39.1)
Midday		24.7 (0.2)	1.67 (0.028)	619.6 (50.6)
Day 35				
Morning	g_s	20.7 (1.2)	0.81 (0.211)	237.8 (62.1)
Midday	g_s	23.0 (0.5)	1.31 (0.097)	432.2 (109.5)
Day 49, midday	g_s	25.6 (0.4)	1.88 (0.094)	511.1 (155.9)
Day 65, midday	g_s	22.6 (0.8)	1.61 (0.108)	868.9 (159.2)
Day 92, midday	ψ_{md}	21.8 (0.3)	0.97 (0.047)	145.8 (12.9)
Day 93, predawn	ψ_{pd}	17.9 (0.1)	0.19 (0.004)	0

5.3.5 Measurements

At the start of the treatment phase, we recorded the following morphological variables: number of leaves (L_n) and branches (B_n), stem length (S_L), stem diameter at base ($S_{D,base}$) and top ($S_{D,top}$), branch length (B_L) and diameter at the base ($B_{D,base}$) and top of the branches ($B_{D,top}$). These measurements were repeated every 3 weeks and at the end of the treatment phase. Total woody volume (V_{tot}, stem + branches) was derived using Smalian's formula (West 2009):

$$V_{tot} = \frac{A_{base} + A_{top}}{2} S_L$$

where A_{base} and A_{top} are the areas at the base and top of the stem or branches, respectively. On days 35, 49 and 65, leaf stomatal conductivity (g_s) was measured on two randomly selected top leaves of all seedlings using a steady-state SC-1 leaf porometer (Degacon Devices, Pullman, WA, USA). These measurements were performed in two rounds between 0900 - 1200 h and 1230 - 1530 h local time on day 35, whereas on the other days g_s was measured only once (between 1230 and 1530 h), since climatic conditions were not homogeneous during the mornings of days 49 and 65. Midday (ψ_{md}) and predawn (ψ_{pd}) leafwater potentials were determined on day 92 (1230 - 1500 h) and 93 (0300 - 0530 h) respectively, using a Scholander pressure chamber (model 615, PMS instruments, Albany, USA). In total, we examined ψ_{md} and ψ_{pd} for 60 seedlings, in each of which two top leaves were randomly selected. These seedlings were characterized by low (≤ 0.67) and high (≥ 0.89) levels of MLH. The water potential range ($\Delta\psi$) per seedling was calculated as ψ_{pd} - ψ_{md}. Climatic conditions during measurements of leaf stomatal conductivity and water potential are given in Table 5.2.

At day 100 of the treatment phase (16 August 2012) all seedlings were harvested and measurements of fresh (W_F) and dry weight (W_D, 48h at 85°C) of the woody parts (stem + branches), leaves, roots and whole plants were performed. We calculated for each seedling above-ground biomass (W_{AG}) and water content (WC) as follows:

$$WC = 100\,(1 - \frac{W_D}{W_F})$$

5.3.6 Calculated variables

To estimate the initial biomass of the seedlings followed during the treatment phase, we established linear regression models between biomass data (W_F, W_D and W_{AG}) and morphological input variables (ln(V_{tot}), ln(S_L), ln(L_n) and B_n), which were obtained from the 50 seedlings harvested at the start of the treatment phase. The models with the highest R^2_{adj} (0.84 - 0.97) were selected for initial biomass estimation (Appendix 5.3). A good model fit was also confirmed by Mallow's C_p selection criterion, as the

C_P value was equal to the number of regressors in the chosen models (Gagné & Dayton 2002). The relative growth rates (RGRs) of various morphological and biomass variables were calculated as:

$$\mathrm{RGR_X} = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

where X_1 and X_2 are the values of the studied variables at times t_1 and t_2 respectively (Evans 1972). Daily transpiration rate (TR) was determined by weighing the pots before and after watering and correcting for soil evaporation (blank pots) and leaf loss. We also accounted for differences in the number of leaves per seedling by dividing the transpiration rate by the ln(L_n) of the seedlings. Transpiration efficiency (TE) was calculated as the ratio between the amount of dry biomass produced during the treatment phase and the total amount of water consumed during this period. We also estimated biomass water productivity (WP), which can be defined as the total dry biomass produced per unit of water transpired, normalized for atmospheric conditions (Steduto 2003; Steduto et al. 2007). Many studies have shown that WP is approximately constant for a given species, regardless of the growth conditions (irrigation treatment), after the variation in atmospheric conditions is normalized (Steduto 2003; Steduto et al. 2007). To calculate WP we first normalized cumulative transpiration (CT) for daily mean VPD measured between 0800 and 1800 h on day j (VPD_j)

$$\mathrm{CT}_i = \sum_{j=1}^{i=100} \frac{\mathrm{TR}_j}{\mathrm{VPD}_j}$$

Secondly, we constructed a linear regression model that included irrigation treatment, the cumulative transpiration normalized for atmospheric conditions (CT_i), MLH and irrigation treatments as input variables, and total dry biomass production as dependent variable. Main effects and interaction terms that were not significant ($p > 0.05$), were excluded from this model using a backward selection procedure. Finally, biomass water productivity was estimated as the regression coefficient of the final regression model with CT_i and irrigation treatment as input variables and dry biomass production as dependent variable (Steduto et al. 2007).

5.3.7 Statistical analyses

We used linear regression models to examine the effects of irrigation treatment (factor), multilocus heterozygosity (covariate) and their interaction term on the response variables measured and calculated during the treatment phase. To test for variation caused by environmental differences between the three seed collection sites, the forest stand in which the acorns were collected was included in the model as a fixed factor. Forest stand was not included as a random effect in our model, since the number of independent clusters within forest stands was too limited (three stands) to properly estimate its standard deviation. We also accounted for the effect of seed size on the measured response variables by including log seed weight in the initial model. Non-significant stand and seed size effects were excluded from the initial models (Appendix 5.4), such that well-fitting final models (highest R^2_{adj}) were obtained. For all tested models, R^2_{adj} values and their significance levels were calculated as a measure of goodness of fit, and partial R^2 coefficients (R^2_p) were obtained for each fixed effect to compare them between the different final models. To examine the effect of time of measurement, repeated measures ANOVAs with between-subject factors forest stand, irrigation regime and MLH were applied to transpiration rates corrected for the number of leaves per seedling (within-subject effect = irrigation event, 39 levels) and to stomatal conductance measured on day 35 (within-subject effect = time of measurement, 2 levels). If the assumption of sphericity was not met (significant Mauchly's test), the Huyn-Feldt statistic was used for within-subject tests. All statistical analyses were performed using SPSS software (SPSS 20.0; SPSS, Chicago, IL).

5.4 Results

A total of 146 of the 150 studied seedlings (97.3%) were successfully genotyped for all nine microsatellite loci. The markers were highly polymorphic, with an average number of 17.7 ± 2.01 (SE) alleles per locus. At the population scale we found high allelic richness and heterozygosity levels for the seedlings ($A_r \geq 11.2$; $H_o \geq 0.794$ and $H_e \geq 0.800$; Table 5.3). Individual multilocus heterozygosity (MLH) ranged between

0.44 and 1, whereas d^2 varied between 9.3 and 751.7 and sMd^2 between 4.3 x 10^{-5} and 8.0 x 10^{-3}. The three variables were significantly correlated with each other. MLH was more strongly correlated to the standardized mean sMd^2 (r_s = 0.39, p < 0.0001) than to the mean d^2 (r_s = 0.27, p = 0.006). In addition, the statistical power of d^2 and sMd^2 to detect significant HFC*s* was much more limited (lower R^2_p values (< 0.1 - 2.1%)) compared with MLH (3 - 11%) (cf. Coltman & Slate 2003; Slate & Pemberton 2002; Chapman *et al.* 2009). Therefore, we only report the outcome of the HFC tests based on MLH.

Table 5.3: Estimates of population genetic diversity measures (± standard errors) for the studied seedlings from three *Q. robur* populations based on 9 microsatellite loci.

Forest stand	A_n	A_r*	Rare alleles (%)	H_o	H_e	F_{IS}
Keffers	12.8 (1.5)	12.7 (1.5)	48.8 (5.0)	0.796 (.037)	0.800 (.029)	0.005 (.025)
Vos	12.2 (1.0)	12.2 (1.0)	50.1 (3.2)	0.794 (.048)	0.806 (.031)	0.015 (.042)
Chartreuse	11.2 (1.2)	11.2 (1.2)	39.7 (3.4)	0.820 (.034)	0.814 (.030)	-0.007 (.032)
Mean	12.1 (0.7)	12.0 (0.5)	46.2 (3.3)	0.803 (.022)	0.807 (.017)	0.005 (0.007)

A_n, *number of different alleles*; A_r, *allelic richness;* H_o, *observed heterozygosity;* H_e, *expected heterozygosity;* F_{IS}, *inbreeding coefficient;*

**allelic richness based on a minimum sample size of 48 individuals*

5.4.1 Water use and transpiration

Almost all transpiration and water use variables strongly differed (p < 0.05) between the two irrigation treatments (Table 5.4A), with significantly higher estimates for seedlings subjected to normal water conditions compared to drought-stressed seedlings (Figures 5.1 & 5.2). Changes in stomatal conductance (g_s) during the day also differed significantly between the two irrigation treatments (significant time x stress effect, p < 0.05). For drought-stressed seedlings, g_s decreased by 9.6% from 67.7 ± 5.8 (SE) mmol $m^{-2}s^{-1}$ in the morning of day 35 to 61.3 ± 5.2 (SE) mmol $m^{-2}s^{-1}$ at midday, whereas g_s of well-watered seedlings significantly increased (p < 0.05) by 32.5% from 337.6 ± 28.5 (SE) to 447.1 ± 44.3 (SE) mmol $m^{-2}s^{-1}$ at midday. More importantly, the results of the linear regression models indicated that g_s and water

content were significantly correlated ($p < 0.05$) with the MLH of the seedlings, whereas ψ_{md} and $\Delta\psi$ showed marginally significant relationships ($0.05 \leq p < 0.1$) (Table 5.4A). For all of these variables, estimates increased with increasing MLH (Figure 5.2), except for $\Delta\psi$, for which the opposite relationship was observed in the drought-stressed seedlings (Figure 5.2D). Although significant HFCs were found, only a small proportion of the variance (R^2_p = 4 - 11%) could be explained by MLH (Table 5.4A). Marginally significant interaction terms ($0.05 \leq p < 0.1$) between irrigation treatment and MLH were obtained for measures of g_s on day 49 and for $\Delta\psi$ (Table 5.4A). These interactions are visualized in the non-parallel regression lines in Figure 5.2A and Figure 5.2D, where estimates of respectively g_s on day 49 and $\Delta\psi$ were more strongly correlated with MLH under water stress than under normal water conditions. The effects of forest stand and seed weight on the transpiration variables were limited. A few variables showed significant differences between the three seed collection sites (Table 5.4), but no effects of seed weight were found.

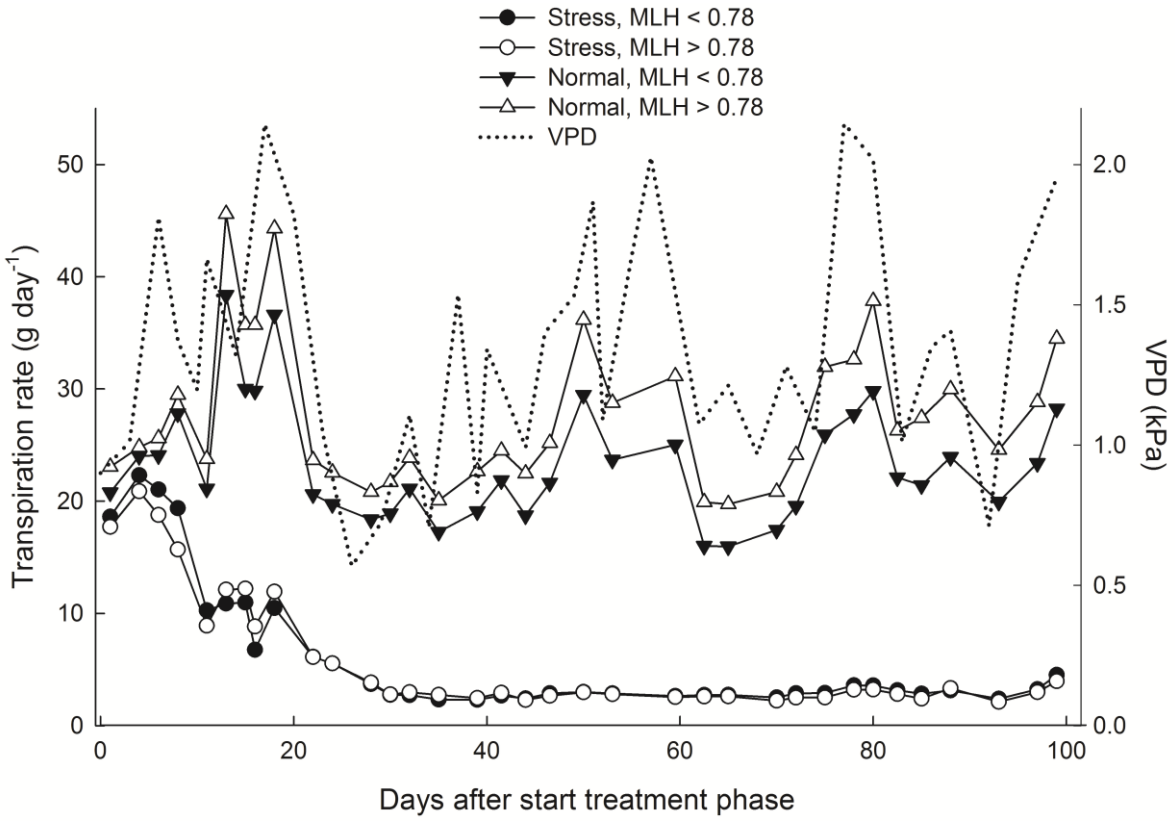


Figure 5.1. Daily transpiration rates of *Q. robur* corrected for the number of leaves per seedling, under drought stress and normal water conditions. Seedlings were divided into two groups with MLH ≤ 0.78 and > 0.78.

Table 5.4. results of the linear regression models performed to examine the effect of irrigation regime, MLH and their interaction on (A) transpiration and (B) growth trait variables for *Q. robur*.

		Correlation model		Irrigation		MLH		Irrigation x MLH		Forest stand		Log seed weight	
Parameter	Variable	F_{model}	R^2_{adj}	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p
(A) Transpiration													
Stomatal conductance	log g_s day35 a.m.	43.03***	0.68	10.27**	0.10	4.76**	0.05	0.36	< 0.01	4.66**	0.09		
	log g_s day35 p.m.	73.79***	0.69	10.02**	0.10	7.13**	0.07	0.26	< 0.01				
	log g_s day49 p.m.	110.81***	0.85	35.55***	0.27	9.06**	0.09	3.20(*)	0.03	5.57**	0.11		
	log g_s day65 p.m.	121.81***	0.86	16.43***	0.15	11.14**	0.11	0.17	< 0.01	2.75(*)	0.06		
Leaf water potential	ψ_{md}	40.35***	0.68	14.01***	0.21	2.81(*)	0.05	1.81	0.03				
	ψ_{pd}	58.93***	0.75	5.87**	0.10	0.10	< 0.01	0.34	< 0.01				
Water potential range	$\Delta\psi$	9.52***	0.31	8.61**	0.14	3.14(*)	0.06	3.93(*)	0.07				
Total transpiration$_{corr}$	TR	111.79***	0.78	3.19(*)	0.03	2.21	0.02	2.69	0.03				
Water content	WC	61.47***	0.66	6.10**	0.06	4.11**	0.04	< 0.01	< 0.01				
(B) Growth traits													
RGR (Evans 1972)	$RGR_{diameter}$	69.40***	0.74	20.06***	0.18	3.67(*)	0.04	2.17	0.02			2.75	0.03
	RGR_{length}	3.58**	0.08	0.03	< 0.01	2.95(*)	0.03	0.45	< 0.01				
	$RGR_{woody\ volume}$	46.26***	0.66	15.21***	0.14	4.53**	0.05	2.22	0.02			2.25	0.02
	$RGR_{dry\ biomass}$	64.86***	0.67	7.55**	0.08	3.23(*)	0.03	0.04	< 0.01				
	$RGR_{fresh\ biomass}$	102.28***	0.76	14.10***	0.13	2.78(*)	0.03	0.28	< 0.01				
	$RGR_{aboveground}$	18.28***	0.48	8.47**	0.09	2.51	0.03	1.76	0.02	5.31**	0.11		
	RGR_{roots}	20.80***	0.56	2.93(*)	0.03	0.12	< 0.01	0.05	< 0.01	6.55**	0.13	4.03**	0.04
Transpiration efficiency	TE	2.41(*)	0.06	0.47	< 0.01	0.05	< 0.01	0.41	< 0.01			8.51**	0.09

To account for the differences between forests and seed sizes, forest stand and log seed weight were included in the analysis as a fixed effect. Non-significant stand and seed effects were excluded from the final models, such that we obtained well-fitting models (highest R^2_{adj}*). F-statistics,* R^2_p *coefficients and significance levels for main effects and interactions are presented for the final models. (*) 0.05 ≤ p < 0.1; ** 0.001 ≤ p < 0.05; *** p < 0.001.*

The repeated measures ANOVA model that was conducted to examine the effect of time on TR indicated a highly significant time (F = 5.39, p < 0.001, R^2_p = 0.06) and time x irrigation effect (F = 63.46, p < 0.001, R^2_p = 0.41) (Figure 5.1). The mean TR of drought-stressed *Q. robur* seedlings declined strongly from 21.6 ± 1.5 g (SE) on day 5 to 3.7 ± 0.1 g on day 28, after which TR stabilized. The seedlings under normal water conditions showed daily TRs that were highly correlated (r_s = 0.72, p < 0.001) to atmospheric demand (VPD), with a slightly, but not significantly higher TR when MLH was > 0.78 compared with seedlings with MLH ≤ 0.78 (Figure 5.1).

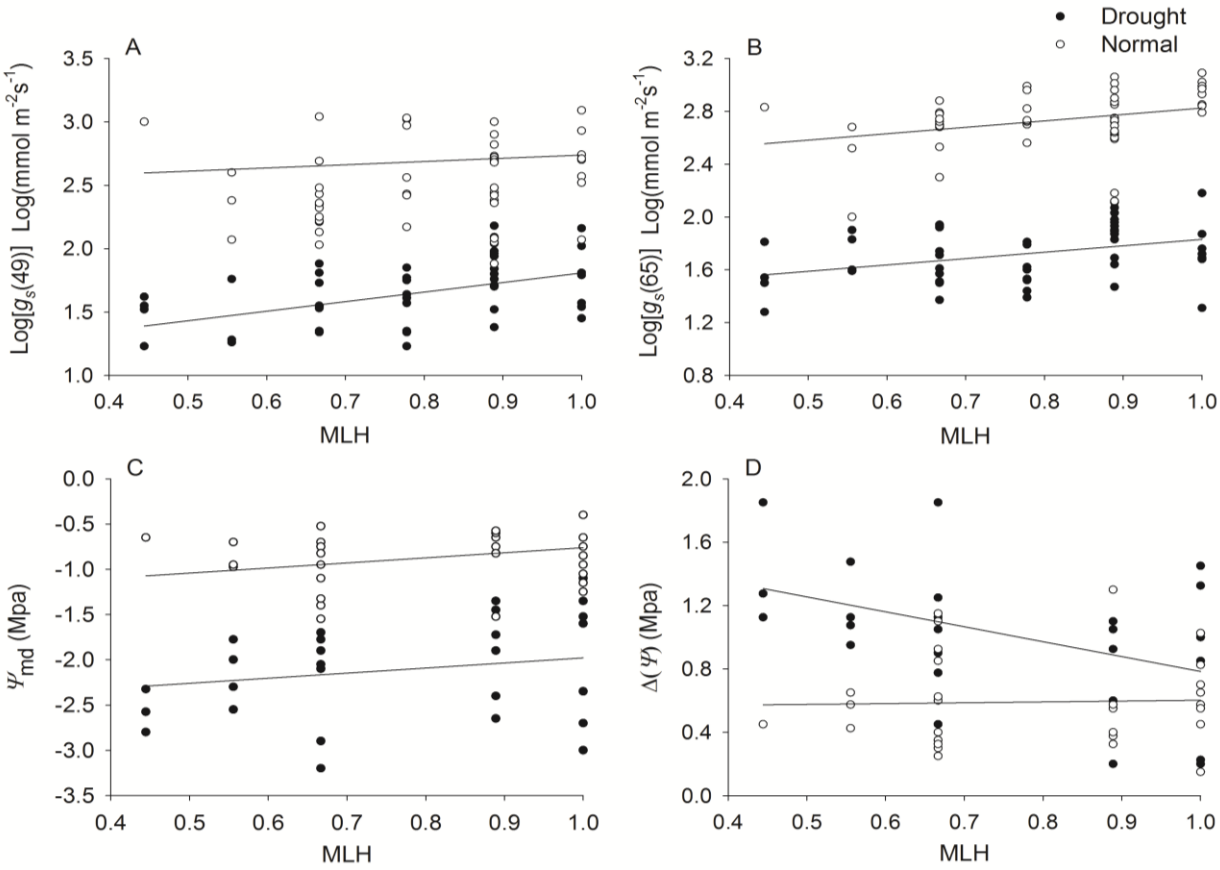


Figure 5.2. Relationships between MLH of *Q. robur* and the following transpiration variables: g_s at (A) day 49 and (B) day 65, (C) ψ_{md} and (D) $\Delta\psi$. HFCs were compared between seedlings subjected to normal water conditions and drought stressed seedlings. The plotted regression lines are based on estimates of the significant fixed effects of the linear regression models (Table 4).

5.4.2 Growth rate and biomass production

Seedling growth was strongly affected by water availability of the soil. First, we observed a highly significant ($p < 0.001$) positive relationship between dry biomass production and the total amount of water transpired during the treatment phase (Figure 5.3). Second, most growth traits were influenced by the irrigation regime, with significantly higher RGRs ($p < 0.05$) under normal water conditions than under drought stress (Table 5.4B). Efficiency in producing a certain amount of dry biomass per unit of water transpired (TE) was 23% higher in drought-stressed seedlings (5.01 ± 0.32 (SE)) than in well watered seedlings (4.07 ± 0.23 SE). However, this difference between irrigation treatments was not significant ($p > 0.05$), and TE was also not associated with the MLH of a seedling (Table 5.4B). Similar results were obtained for biomass WP, since the regression coefficients of the linear regression model between CT_i and dry biomass production were not significantly ($p > 0.05$) influenced by the other input variables of the model (irrigation treatment and MLH). This was indicated by the non-significant interaction terms (CT_i × irrigation treatment and CT_i × MLH), which were removed, together with the non-significant main effect MLH, from the linear regression model.

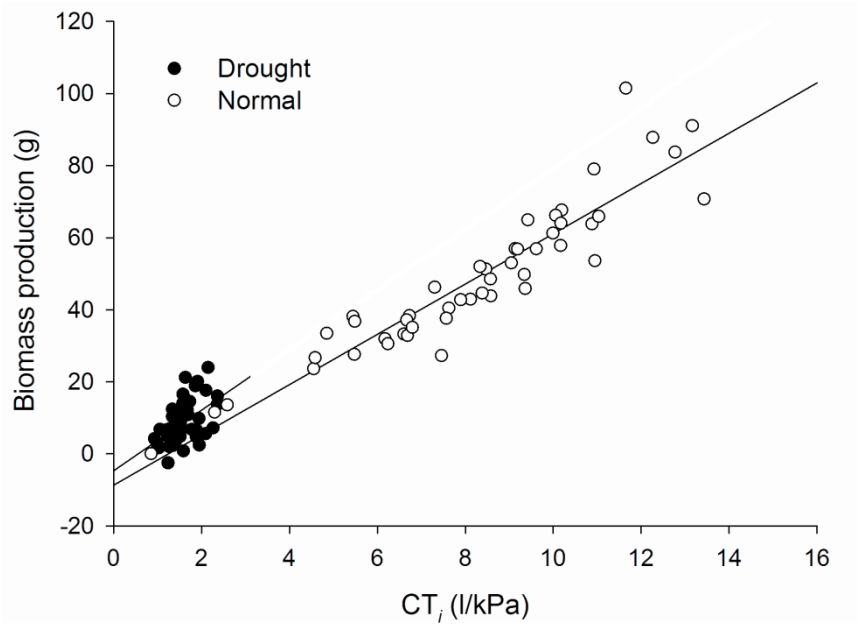


Figure 5.3. Biomass production of *Q. robur* as a function of cumulative transpiration standardized for VPD (CT_i), compared between seedlings under normal water conditions and drought-stressed seedlings. Regression lines are plotted for each irrigation treatment and are based on the coefficients of the linear regression model.

A significant effect of MLH ($p < 0.05$) on growth variables was obtained when the RGR of woody volume was tested as trait (Table 5.4B). Under drought stress, mean values of $RGR_{woody\ volume}$ were 7.5 times larger in seedlings of the highest MLH class (MLH = 1) compared with seedlings with the lowest MLH values (MLH = 0.44) (Figure 5.4A). The RGR of stem diameter and RGRs of dry and fresh biomass showed a weak (R^2_p = 3 - 5%), marginally significant ($0.05 \leq p < 0.1$), positive relationship with MLH (Table 5.4B, Figure 5.4B). In contrast to some of the transpiration variables, no significant interaction terms between MLH and irrigation treatment were found for any growth trait (Table 5.4B, Figure 5.4). Finally, most of the growth traits were not significantly influenced by the forest stand in which the acorns were collected or by the weight of the acorns. Furthermore, the weight of the acorns was not significantly correlated with the MLH of the acorns ($r_s = 0.12$; $p = 0.23$).

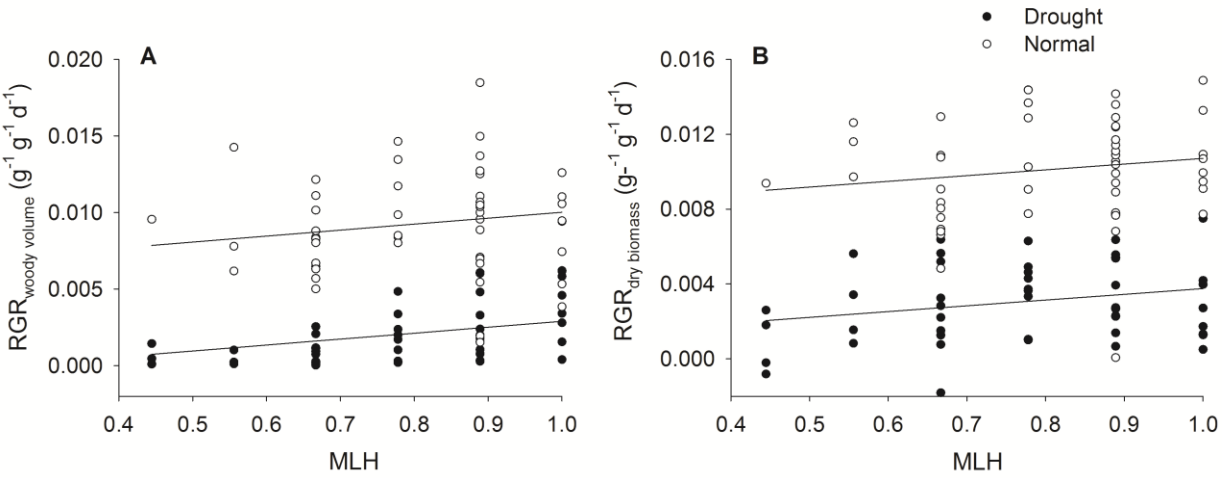


Figure 5.4. Relationships between MLH of *Q. robur* and (A) RGR of woody volume and (B) RGR of dry biomass. HFCs were compared between seedlings subjected to normal water conditions and drought-stressed seedlings. The plotted regression lines are based on estimates of the significant fixed effects of the linear regression models (Table 5.4).

5.5 Discussion

Our results showed weak but significant effects of MLH on several transpiration and growth traits in the temperate broadleaved tree species pedunculate oak (*Quercus robur*). Since selectively neutral molecular markers were used to quantify genetic diversity, HFCs cannot readily be attributed to functional overdominance at the

scored loci (the so-called direct effect hypothesis) (Queller et al. 1993; Savolainen and Hedrick 1995), but they would require small non-random mating population structures to emerge. In these populations, processes such as genetic drift, inbreeding and recent bottlenecks are more likely to occur (Young et al. 1996), and consequently HFCs may arise as a result of associative overdominance, either at linked fitness loci (the local effect hypothesis) or at genome wide distributed loci (the general effect hypothesis; i.e. classical inbreeding depression) (Szulkin et al. 2010). Although *Q. robur* is a wind-pollinated species with a highly outcrossing breeding system (selfing rate 2 - 5%; Steinhoff 1993), it has been shown that, in tree species with similar traits, biparental inbreeding may lower the number of heterozygous individuals in small, fragmented forest stands (Jump & Peñuelas 2006; Vranckx et al. 2012). Furthermore, recent research conducted in the same forest stands by Vranckx et al. (2014) has demonstrated less diverse pollen pools and increased correlated paternity in small stands. Ultimately, this may lead to stronger effects of biparental inbreeding in subsequent generations. In this study some variation in MLH was already detected for individual seedlings. However, at the population scale we found high heterozygosity levels (Table 5.3), which were consistent with what was found in other population genetic studies on *Q. robur* (Mariette et al. 2002; Hampe et al. 2010).

5.5.1 Strength of HFCs in *Q. robur* seedlings

The observed proportion of the variance in transpiration and growth traits that was explained by MLH was substantially larger than that reported in earlier HFC studies (0.07 - 3.3%, Szulkin et al. 2010). Furthermore, explained variances of ≤ 1% for MLH have been reported to be common when microsatellite markers are used to quantify genetic diversity (Coltman & slate 2003; Chapman et al. 2009). The stronger HFCs that we obtained can be attributed to several factors. First, since most empirical HFC studies have been performed on animal species with separate sexes (Coltman & Slate 2003; Chapman et al. 2009; Szulkin et al. 2010), significant publication bias is likely. Plant species can be expected to show stronger HFCs than animal species, which may be related to the sessile nature and often self-compatible breeding systems of plants. This makes them more prone to selfing and biparental inbreeding, especially when

habitat fragmentation occurs (González-Varo et al. 2012). Second, the choice of genetic metric may also influence the strength of the HFC (Slate & Pemberton 2002; Chapman et al. 2009). Our study indicated that MLH had a higher power to detect HFCs than the variables d^2 and sMd^2. This is consistent with the results of previous meta-analyses (Coltman & Slate 2003; Chapman et al. 2009) and may indicate the occurrence of recent inbreeding events rather than population admixture (Coulson et al. 1998; Pemberton et al. 1999). As already mentioned, d^2 may show stronger HFCs than MLH when population admixture strongly increases the number of highly heterozygous individuals and when fast-evolving molecular markers (mutation rate > 0.001) are used (Goudet & Keller 2002). Third, contrary to previous studies in conifers (Ledig et al. 1983; Savolainen & Hedrick 1995), we examined *Q. robur* seedlings under controlled common garden conditions, which increased the probability of detecting HFCs (David & Jarne 1997; Keller & Waller 2002). Heterozygosity–fitness correlations are expected to be stronger in seedlings compared with adult trees, as growth and survival are most strongly affected during the earliest life stages (David 1998). Adult trees may cope better with stressful conditions such as drought stress as their roots may access deeper water sources, whereby higher rates of transpiration and photosynthesis are maintained (Bond 2000; Cavender-Bares & Bazzaz 2000). Furthermore, less fit homozygotes, still present in the seedling cohort, may be absent from older generations (Honnay et al. 2008). Because of this gradual decrease in the number of homozygous individuals from seedlings to adult trees and the reduced environmental stress perceived by the adults, one would expect that under natural conditions HFCs would have been absent or undetectable in adult cohorts. However, Ziehe and Hattemer (2002) still found positive associations between heterozygosity level and diameter growth of adult trees in natural populations of the temperate broadleaved tree species *Fagus sylvatica*.

Perhaps the most important factor influencing the magnitude of HFCs is the fitness trait under study. In our study, transpiration and growth traits showed (marginally) significant relationships with MLH. HFCs were, however, stronger for

transpiration variables (R^2_p = 4 - 11%) than for growth traits (R^2_p = 3 - 5%). When *Q. robur* seedlings are facing drought stress, stomatal conductance is directly reduced as a result of a highly efficient stomatal control mechanism. This allows the leaf water potential to remain above the critical threshold value at which cavitation damage occurs (Vivin et al. 1993; Cochard et al. 1996). Contrary to the growth traits, which were recorded over a longer period of time, the transpiration variables were measured at midday (1230 - 1530 h). Consequently, transpiration was strongly affected, as the seedlings were exposed to the highest possible values of VPD and high levels of PAR (Table 5.2). Similar midday depression of gas exchange has been reported frequently in oaks and many other tree species (Weber & Gates 1990; Epron & Dreyer 1993) and was confirmed in this study by the decreased midday stomatal conductance (29.6%) of the drought-stressed seedlings on day 35.

Another possible explanation for the stronger correlations between transpiration variables and MLH is that these HFCs could be the result of an association with a microsatellite near the coding region of the studied trait (the local effect hypothesis). Although theoretical papers often suggest that transpiration and growth traits are both typically controlled by numerous loci (David 1998; Szulkin et al. 2010), recent research has detected a relatively limited number of quantitative trait loci (QTLs) for growth rate and several transpiration traits in *Q. robur* (Scotti-Saintagne et al. 2004; Brendel et al. 2008; Gailing et al. 2008). For example, in the study of Scotti-Saintagne et al. (2004) three QTLs for height growth were found which explained between 9.5 and 18.7% of the mean variance. One of the problems of QTLs is that they may be specific to a given environment, growth stage or genetic background, because of which QTL consistency across studies is often low (Teulat et al. 2001). Only in the study of Gailing et al. (2008), were some of the microsatellites used in our study located within QTLs for height growth and stomatal density. For height increment, two microsatellites (QpZag 104 and QpZag 46) were positioned within one QTL region (LG2M), explaining 3.4% of the variance in height growth, whereas for stomatal density three microsatellites (QpZag 104, QpZag 46 and QpZag 9) occurred within two QTLs (LG2F and LG7F), explaining a higher percentage (3.7

and 7.2%, respectively) of the phenotypic variance (Gailing et al. 2008). It has been shown that increased stomatal density may improve drought resistance, as stomata present at high density are often smaller and have small guard cells, which contributes to better control of transpiration (Roussel et al. 2009).

The local effect hypothesis was, however, not supported by the Ewens-Watterson homozygosity test of neutrality (Manly, 1985) and the F_{st} outlier test (Excoffier et al. 2009), as both tests showed no significant departures from neutrality for all microsatellites. Moreover, when we removed locus QpZAG104 or QpZag46 from our dataset, similar results were obtained (appendix 5.5 and appendix 5.6) as in the primary analyses without exclusion of QpZAG104 or QpZag46 (Table 5.4). We tested the removal of both loci, as QpZAG104 was close to the lower limit of the 95% confidence interval of the Ewens-Watterson homozygosity test, and since they were reported in the study of Gailing et al. (2008) to be located within QTLs for height growth and stomatal density.

5.5.2 Interaction between drought stress and heterozygosity

It has been claimed that HFCs are more pronounced under elevated environmental stress levels than under optimal conditions (Armbruster & Reed 2005; Lesbarrères et al. 2005), possibly exacerbating climate change effects in small, inbred tree populations. In our study, little evidence was found to support this hypothesis, as most of the examined traits showed no significant interaction between irrigation treatment and MLH. However, for the water potential range, we found stronger HFCs in drought-stressed seedlings compared with well watered seedlings. Plants can recover from water deficits overnight through internal hydraulic redistribution, which removes water potential gradients among leaves and roots (Domec et al. 2004; Bauerle et al. 2008).This will alleviate plant water stress, as root function, cell turgor for growth and leaf water content are maintained (Nardini & Pitt 1999). The variation in recovery rate of the water potential that was observed in the drought-stressed seedlings was influenced by the level of midday leaf water potential, which, in turn was affected by the individual MLH.

Next to the limited effect of the irrigation treatment, the strength of the HFCs may also have been influenced by the atmospheric stress level. Stomatal conductance was more correlated with MLH on days 49 and 65, which were both characterized by high VPD and PAR levels (Table 5.2), whereas on day 35 there were lower atmospheric stress levels and weaker HFCs (Table 5.4). Previous studies on tree transpiration have indicated not only that the physiological response of trees to drought stress depends on the water status of the soil, but also that the VPD and PAR level may also play a major role in transpiration and growth (Van Hees 1997; Oren & Pataki 2001; Zweifel et al. 2005). The significant interaction between irrigation treatment and MLH that was observed on day 49 might be attributed to the combined effect of low soil moisture and high atmospheric stress (e.g. high VPD and PAR), which may have imposed stronger overall drought stress conditions on seedlings (Van Hees 1997). This is in contrast with the findings of Mitton (1993) and Audo & Diehl (1995), who found stronger HFCs at moderate stress levels, whereas we demonstrated that higher stress levels (especially atmospheric stress) exacerbated the effects of low genetic diversity on tree transpiration.

5.5.3 Implications for future forest management under climate change

The existence of HFCs in natural populations of pedunculate oak indicate that increased homozygosity could ultimately limit biomass production below the potential yield. We found that relative growth rates of biomass production and woody volume declined with increasing number of homozygous loci under both irrigation treatments. Considering ongoing climate change, the projected increase in temperature of 2.0 - 3.1 °C for Central Europe by 2100 (CMIP5 model, scenario RCP4.5, 25^{th} - 75^{th} percentile), will raise the VPD of the air by 3 - 6% $°C^{-1}$ (Kirschbaum 2000; Stocker et al. 2013), leading to greater exposure of trees to atmospheric drought stress. Since we have shown stronger relationships between transpiration rates and MLH under higher VPD levels and since biomass production was strongly correlated with total transpiration, one can expect lower biomass production in homozygous individuals in the future. Moreover, more frequent and severe drought events may also limit the water availability in the soil, which may worsen the effect of high levels

of atmospheric stress (Kirschbaum 2000; Zweifel et al. 2005). In our study, however, the effect of limited soil water availability on the strength of the HFCs was only observed for measurements of stomatal conductance on day 49 (highest VPD), indicating a large tolerance of pedunculate oak seedlings to soil water stress (Van Hees 1997; Bréda et al. 2006). Since the roots of adult trees may tap into deeper water sources, they will be even less susceptible to soil water stress than seedlings (Bond 2000; Cavender-Bares & Bazzaz 2000). Furthermore, the drought stress conditions that were established under greenhouse conditions, were not totally representative for those prevailing in the field. One can expect higher drying rates in pots compared to the situation in the field. However, under more natural conditions, the effect of drought stress could be worsened by other stresses not examined in the present study such as frost, fungal infections and insect attacks.

To narrow the gap between average and potential yields under current and future environmental conditions, individual MLH should be maximized in forest tree breeding programmes. In naturally regenerating forest stands, intense natural selection at the seedling stage may preserve high levels of MLH in older age classes (Bush & Smouse 1991). However, natural regeneration of oak is often problematic due to factors such as low seed quantity and quality, strong predation and the lack of appropriate site conditions (Lorimer 1992; Abrams 2003). Moreover, in small fragmented forest stands, which are common in many parts of Western Europe, inbreeding may reduce the number of seedlings with high MLH. Extensive gene flow between stands may mitigate loss of genetic diversity. However, we previously showed that even in wind-pollinated species, such as oak, a reduction in tree density or population size may decrease local pollen diversity and increase correlated paternity (Vranckx et al. 2014), which may lead to stronger effects of biparental inbreeding in subsequent generations. So, even under high pollen immigration rates from outside the stand, an important fraction of mating events will occur at short distances between neighbouring trees (Sork et al. 2002; Breed et al. 2013b). Processes such as biparental inbreeding and reduced gene flow may be avoided by the maintenance of large continuous forest stands (Jump & Peñuelas 2006; Vranckx et al. 2012). Not only may this increase MLH, but it will also retain and enlarge the gene

pool for adaptation, which is probably the best strategy to counter current and future environmental changes (Hamrick 2004; Jump et al. 2009).

Chapter 6.

General Discussion

6.1 Outline of main results

Given the rising public concern regarding forest fragmentation and loss of forest biodiversity, forest policy increasingly promotes a sustainable and multifunctional forest management with a strong focus on the conservation of forest biodiversity. Nevertheless, despite the field of forest landscape genetics has been an attractive science domain throughout the past decades, our understanding of the consequences of anthropogenic disturbance for genetic diversity of tree populations remains limited (Kramer et al. 2008). A general framework to assess the long term sustainability of fragmented forests in terms of tree genetic diversity, through integrating stand characteristics, species traits, population genetics and individual tree performance, is largely lacking in the literature.

The general aim of this study (**chapter 1**) was to help resolving the debate on forest fragmentation genetics by providing increased insights into the effects of human land use and forest management activities on the genetic diversity, gene flow and inbreeding of oak populations. In order to achieve this aim, a multidisciplinary approach was adopted. First, a systematic and quantitative review of the literature on forest landscape genetics was performed. This allowed us to determine the extent to which tree species are at risk to lose genetic diversity following fragmentation of tree populations, and to evaluate how this genetic response was mediated by several life-history traits (**chapter 2**). The results of this meta-analysis were compared with what was found in a more in-depth study on pedunculate oak (*Quercus robur*), where we examined how much of the total genetic diversity was maintained in the naturally established offspring cohort within small oak stands (**chapter 3**). We also investigated the effects of different forest stand characteristics on this process, and how these affected mating patterns and gene flow (**chapter 3 & 4**). Finally, the relationships between tree genetic diversity and tree performance were explored in a large-scale greenhouse experiment (**chapter 5**). The main results of this PhD thesis are schematically presented in figure 6.1.

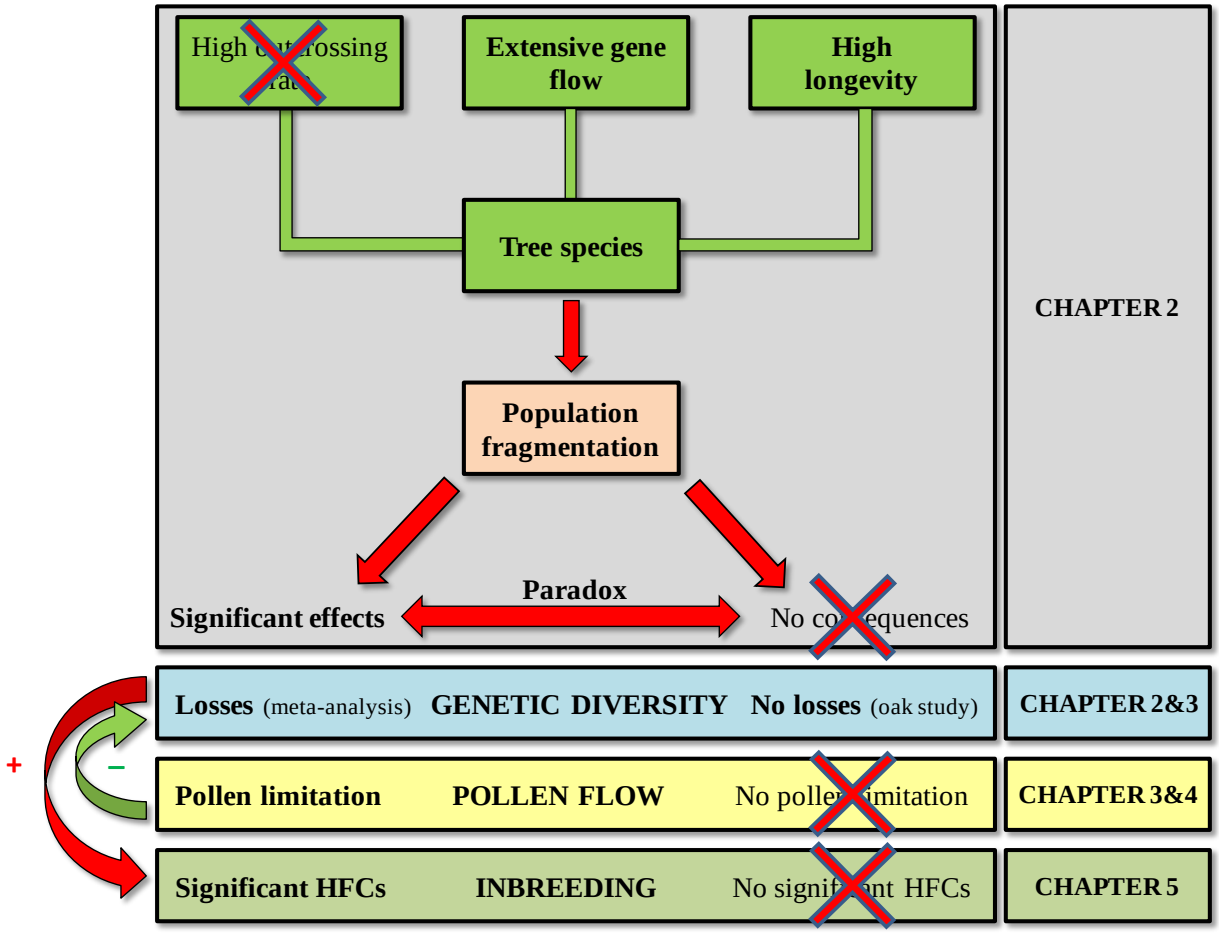


Figure 6.1: Schematic overview of the main results of this thesis, based on a meta-analysis (**chapter 2**) and on a more in-depth study on pedunculate oak (*Quercus robur*) (**chapter 3,4 & 5**).

Our meta-analysis clearly showed that woody plant species were as prone to genetic erosion through habitat fragmentation as herbaceous species studied in earlier meta-analyses (Honnay & Jacquemyn 2007). This suggests that the life history traits that are typically associated with tree species (e.g., high outcrossing rates and extensive gene flow) did not alleviate the negative genetic consequences of forest fragmentation. On the one hand the genetic diversity of obligate outcrossing trees was as strongly affected by habitat fragmentation as self-compatible tree species. This can be explained by the fact that even in self-compatible trees mating will be primarily outcrossing and that, no predominantly selfing woody species are known (Hamrick & Godt 1996). On the other hand, wind-pollinated tree species were as likely to lose genetic diversity as insect-pollinated species. So, also in wind-pollinated tree species which are presumed to have extensive pollen flow, pollen limitation may occur when the scale of spatial isolation between forest fragments exceeds the scale

of pollen flow. Although bird-pollinated species showed no significant genetic effects of habitat fragmentation, one could wonder whether this was due to long-distance pollination events by birds (Murcia 1996; Kramer et al. 2011), or if this resulted from the underrepresentation of studies that examined bird-pollination in our meta-analysis. As a consequence of the latter, the statistical power to find significant fragmentation effects was much lower for bird-pollinated tree species (only 13 data points) than for wind- and insect-pollinated species (37 and 54 data points respectively). Moreover, almost all bird-pollinated species in our meta-analysis were distributed throughout the tropics. This could have provoked stronger adaptations to long-distance gene flow due to the lower tree densities during their evolutionary history (White et al. 2002). Our study also confirmed that tree species, because of their long lifespan, may persist in forest remnants for a long time, without showing strong genetic losses (Hamrick 2004). Only studies that investigated individuals that were exposed to habitat fragmentation for several generations showed significant genetic responses. This implies that recently fragmented or recently planted small forest stands may lose some of their genetic diversity over time.

Contrary to the above meta-analytical findings, no strong effects of anthropogenic disturbances on genetic diversity were observed in our in-depth study on pedunculate oak (**chapter 3**). However, all studied oak stands were representative for the typically strongly fragmented forest landscape of North-western Europe (forest stand sizes < 5ha). The lack of clear differences between the genetic diversity measures of the recently established seedling cohorts and the adult generations in these small and low-density forest stands could be attributed to several factors. First, as tree planting in Flanders was (and still is) a common silvicultural practice (Tack et al. 1993), and since the adult generation did not show a significant spatial genetic structure (SGS) in all study plots, this suggested that the studied oak stands probably originated from recent tree planting (< 200 years). Given the long generation time of oak, this might imply that the genetic diversity of the standing population did not yet reflect that of the current small and fragmented forest stands.

The maintenance of high levels of heterozygosity and allelic richness in the progeny cohort could also be explained by large effective population sizes (N_e), which, in turn, could be ascribed to extensive pollen flow in the *Q. robur* stands. Indeed, since the forest stands that were studied in **chapter 3** were relatively limited isolated (135 - 1000m) from the nearest *Q. robur* stand, pollen immigration from outside the stand could have mitigated the negative genetic consequences associated with small and low-density forest stands. Although high out-of-plot pollen immigration rates were found in all study plots, only in the stand Chartreuse where all adult trees were sampled, we were sure that these estimates were entirely due to long distance pollination events from outside the forest stand. The other study plots that were studied in **chapter 3 and 4**, were surrounded by a large number of ungenotyped adult trees, through which out-of-plot pollen immigration caused by these ungenotyped adults could not be differentiated from long distance pollination events from outside the oak stands. The complete sampling of all candidate parents within a forest stand would have given more insight in the proportion long distance pollination events that contributed to the offspring cohort.

Nevertheless, even under high out-of-plot pollen immigration rates, an important fraction of mating events occurred at short distances between neighbouring trees (**chapter 4**). Consequently, when the number of adult trees within a forest stand is too strongly reduced, the larger average effective pollination distances may not totally compensate for the reductions in population size, and pollen limitation may occur (Sork & Smouse 2006). Also in our study plots the relatedness of the seedlings and the degree of pollen differentiation were significantly correlated to the size and density of the forest stands. This suggests that decreasing the number of local mating partners within a forest stand may lead to less diverse local pollen pools, which increases the likelihood of inbreeding in subsequent generations. One should notice, however, that we collected offspring samples that originated from seeds produced during one mast year, while tree reproduction can be spread over many seasons. Consequently, due to stochasticity and variation in environmental factors that affect the mating system and gene flow of *Q. robur*, the composition of the local pollen pool may vary across years (Petit & Hampe 2006).

Regardless of the strongly restricted recruitment window of pedunculate oak, it would be beneficial to sample seedlings over several seasons to get a better idea of seasonal fluctuations (mast vs. normal seed years) in gene flow and genetic composition of the seedlings.

Despite the high heterozygosity levels that were found at the population scale (**chapter 3**), a wide variation in multilocus heterozygosity (MLH) was detected for the individual seedlings. This variation was a prerequisite to explain the significant heterozygosity-fitness correlations that were detected for several transpiration and growth traits in our study (**chapter 5**). One could wonder, whether this variation in MLH was due to non-random mating population structures within the forest stands, or whether this variation was normal and resulted from growing seeds under optimal conditions in a greenhouse, through which they potentially carried a large genetic load. Since clear effects of genetic drift and inbreeding on genetic diversity were not yet observed in the offspring cohort of the studied stands (**chapter 3**), the latter explanation seems the most likely. Whether or not the observed HFCs resulted from inbreeding, it was particularly striking that we were able to demonstrate the existence of weak but significant HFCs, as this usually requires much larger sample sizes and a larger number of microsatellites than used in our study (Balloux et al. 2004; Slate et al. 2004; Szulkin et al. 2010). Contrary to earlier HFC studies in coniferous trees, which yielded null results (Savolainen & Hedrick 1995), several sources of variation (seedlings versus adults, greenhouse versus field conditions) were removed in our study, which increased the likelihood of detecting HFCs. The importance of these HFCs could, however, be questioned under field conditions. Under natural conditions, high variability in habitat quality will have a greater impact on fitness than heterozygosity, while selection against homozygous individuals will reduce variation in MLH over time. Consequently, HFCs in natural populations may become increasingly undetectable from the seedling cohort to the adult generation.

Besides detecting significant HFCs, our study was one of the first that found evidence for the detrimental interaction between increased drought stress and

decreased genetic variation on tree performance. These interactions were only found for transpiration variables under high atmospheric stress levels (e.g. high VPD and PAR), which suggests that only above a certain threshold level, the detrimental effect of environmental stress on HFCs may become apparent (Fox & Reed 2011). Since pedunculate oak possesses a highly efficient stomatal control mechanism, oak seedlings directly respond to high drought stress levels by inducing stomatal closure (Fort et al. 1997; Cavender-Bares & Bazzaz 2000). Although this mechanism allows the avoidance of cavitation damage, it will also entail important costs due to reduced photosynthesis and biomass production (Bréda et al. 2006). This indicates the necessity to maximize the individual MLH in natural populations, especially in view of climate change which will raise the VPD of the air in the future. Moreover, since forest fragmentation and habitat degradation will cause changes to various environmental conditions, environmental stress may occur under a wide variety of conditions, through which the genetic consequences of forest fragmentation are further exacerbated.

6.2 Evaluation of current and future genetic risks faced by fragmented pedunculate oak stands

High levels of within-population genetic variation in combination with strong and variable selection at the seedling stage may offer pedunculate oak a great potential for adaptation (Petit & Hampe 2006). To allow rapid adaptation to changing environmental conditions, it is therefore essential that the evolutionary potential of *Q. robur* is safeguarded through maintenance of genetic diversity, through transmitting it to future generations (Jump et al. 2009). Since, at first sight, no clear losses of genetic diversity were observed across generations in the studied pedunculate oak stands, one could assume that the estimated effective population sizes (N_e = 22.6 - 58.4 individuals) were sufficient to avoid the negative impacts of genetic drift and inbreeding. Furthermore, the fragmented forest stands showed high heterozygosity levels that were consistent with what was found in other population genetic studies on *Q. robur* (Mariette et al. 2002; Hampe et al. 2010). Such high levels

of genetic diversity can be attributed to the specific life history traits of pedunculate oak (long-distance pollen flow, strong selection at the seedling stage), which promote the presence of outcrossed adults and the maintenance of genetic diversity in later life stages (Husband & Schemske 1996; Kaufmann et al. 1998).

On the long term, however, these small and fragmented oak stands are not necessarily safeguarded against the erosion of evolutionary potential. When we calculated the average number of generations until the loss of rare alleles (Figure 6.2; Kimura & Ohta 1969), alleles with a very low frequency (< 0.01) may be lost relatively rapidly (within 1.5 - 4 generations) from populations with a N_e ranging between 20 and 60 individuals. These low-frequency alleles are, however, important for the long-term response to selection and rapid adaptation to changing environmental conditions (Lesica & Allendorf 1992). Furthermore, based on the estimates of contemporary geneflow, which will more directly respond to heterogeneity and anthropogenic changes in a landscape, we found some evidence of increased pollen limitation in small and low-density forest stands. Therefore to mitigate the potential negative genetic consequences through increased biparental inbreeding in subsequent generations and to maintain rare, potentially adaptive alleles, in small

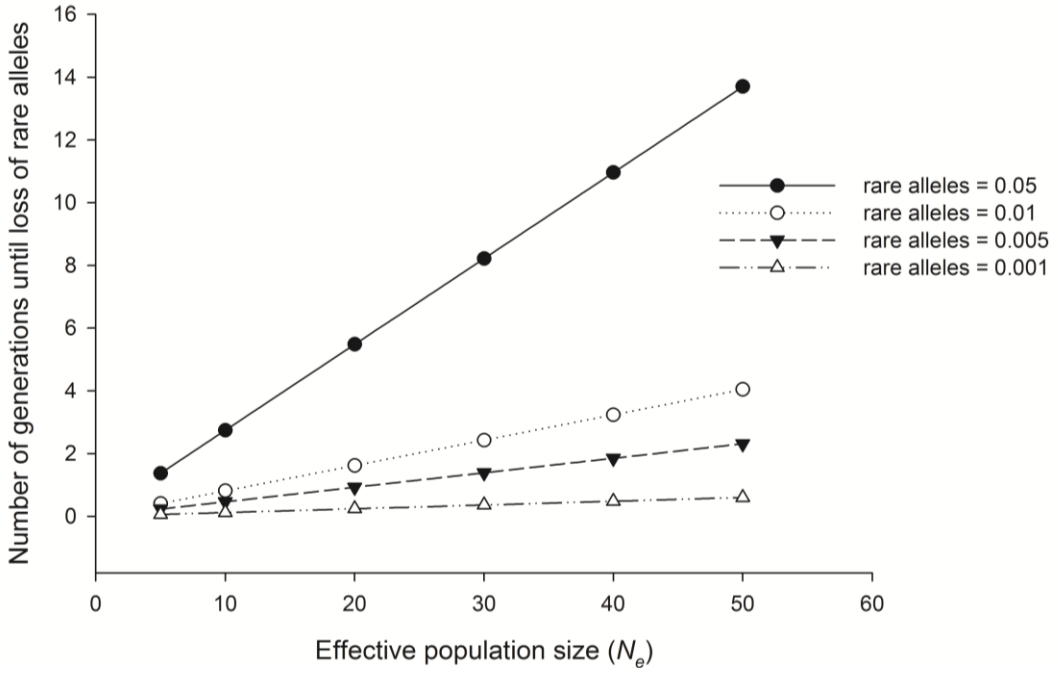


Figure 6.2. Number of generations until loss of rare alleles plotted against effective population size (N_e) (Kimura & Ohta 1969).

fragmented *Q. robur* stands, effective population sizes should be maximized in the seedling cohort. Selecting an appropriate regeneration strategy for pedunculate oak is crucial in this respect.

In a context of close-to-nature forest management, with a strong emphasis on the conservation and sustainable use of forest products, natural regeneration of pedunculate oak has been strongly encouraged in Flanders and in many parts of Western Europe (Forest Europe 2011). From an evolutionary point of view, natural generation is generally considered as an effective way to maintain the genetic diversity and evolutionary potential of trees (Finkeldey & Ziehe 2004). Especially in pedunculate oak, which is a light-demanding and masting tree species natural selection will be efficient. This is because large numbers of seeds are produced during masting years (> 30,000 seeds per seed tree, Bary-Lenger & Nebout 1993), and because seedlings require high irradiance and thus forest canopy opening for their growth and development (Vera et al. 2006). As a result mortality rates of seedlings can be very high in naturally regenerated pedunculate oak stands, allowing permanent natural selection and favoring well adapted progeny.

Apart from this great potential for adaptation, the reproduction-effective population size of natural regenerated oak forest stands can be influenced by stand characteristics such as population size, stand isolation and tree density (Sork et al. 2002; Fernández-Manjarrés & Sork 2005). Also in our study we found some evidence of increased pollen limitation in small and low-density forest stands. Consequently, it is recommended to adapt the classic shelterwood system that is often applied in naturally regenerated pedunculate oak forests into a more passive shelterwood regeneration system. In a classical shelterwood system, overstory trees are removed in a series of cuts to prepare natural regeneration ("preparation and establishment cut"), which will directly reduce tree density and the effective population size. As we have shown, decreased tree density may lower the number of local mating partners and will lead to less diverse pollen pools. Ultimately, this may increase the likelihood of consanguineous mating and inbreeding in the next generation (Young et al. 1996). By postponing regeneration cuts until natural regeneration has established, a larger

proportion of adult trees will genetically contribute to the next generation (Finkeldey & Ziehe 2004).

Another more important factor that may increase the effective population size in small fragmented natural regenerating oak stands is extensive pollen flow (Dow & Ashley 1998). In oak species, pollen may travel up to several kilometres, through which they are assumed to be resilient to reproductive isolation resulting from habitat fragmentation (Craft & Ashley 2010; Buschbom et al. 2011). In this respect, small pedunculate oak stands may make an important contribution to the genetic connectivity across the landscape, as they may serve as stepping stones for long-distance pollen dispersal within fragmented forest landscapes. However, even in a wind-pollinated tree species such as pedunculate oak, the amount of gene flow among remnant populations will depend on the degree of geographical isolation between habitat fragments, the quality of the intervening landscape matrix and the diversity of pollen and seed sources that contribute to the local gene pool (Sork et al. 2002; Bacles & Jump 2011). Small forest fragments that are strongly isolated and that have a low diversity of contributing pollen, may have an increased risk for pollen limitation (Sork & Smouse 2006). Also in our study plots, we found some evidence of increased pollen limitation in small and low-density forest stands, which suggested that the negative genetic consequences associated with a restricted number of local mating partners were not totally compensated by long-distance pollen flow from outside the forest stands. Although conservation resources should be directed to establish genetic connectivity across fragmented forest landscapes, this is not always realistic in the highly fragmented forest landscapes of Northern Belgium and other parts of Europe (Wiersum et al. 2005). Nevertheless, since small pedunculate oak stands may make an important genetic contribution to the effective breeding population in fragmented forest landscapes, their evolutionary potential should be protected.

Supplementation of natural regeneration by sowing or enrichment planting may increase the diversity of pollen donors contributing to the progeny in small pedunculate oak stands. The forest reproductive material used for this purpose

should preferably be harvested in one or several large, continuous and higher-density forest stands, to avoid the collection of inbred seed material. When inbred seeds are raised under optimal conditions in a greenhouse or nursery, they may still carry a large genetic load. As we have shown in our study, this may affect individual tree fitness through influencing growth and transpiration variables, and could ultimately result in a decreased performance of artificial regenerated trees under heterogeneous field conditions. Also the transfer of reproductive material that originate from different provenance regions could be applied to enlarge the evolutionary potential to current and future environmental changes (Broadhurst et al. 2008; Breed et al. 2013a). Traditionally, local seed provenances were chosen over multi-provenance seed material for regeneration purposes, as the latter may increase the risk for maladaptation and outbreeding depressions (Templeton 1986, Lynch 1991). Nevertheless, given the current and future predicted climatic changes, the migrational capacity of pedunculate oak may be too limited, through which local genotypes are at risk of being maladapted to future conditions (Breed et al. 2013a).

To keep pace with rapidly changing environmental conditions, assisted migration through combining the benefits of local and more distant seed sources (composite provenancing) have been increasingly applied in regeneration and reforestation programmes (Breed et al. 2013a). Composite provenancing will primarily source seeds from local provenances near the target site, but will also include a small proportion of material from more distant sources (Broadhurst et al. 2008). This may facilitate the production of new gene complexes in later generations when local and distant genetic material is crossed. On the one hand this may lead to increased seedling failure when the distant material is maladapted or as a result of outbreeding depression. Nevertheless, this risk is relatively negligible on the population scale, as only a small proportion of material from distant sources is used for regeneration. On the other hand, some later-generation hybrids may have higher fitness and may be better adapted to current or changed environmental conditions (heterosis) than the original parental populations (Erikson & Fenster 2006). Subsequently, these hybrids may be rapidly spread throughout the population through natural selection. In conclusion, selecting the appropriate regeneration

strategy for pedunculate oak (natural or artificial regeneration, or both) will be best determined by its likelihood to maintain genetic diversity and will consequently strongly depend on the characteristics of the forest stand (Figure 6.3).

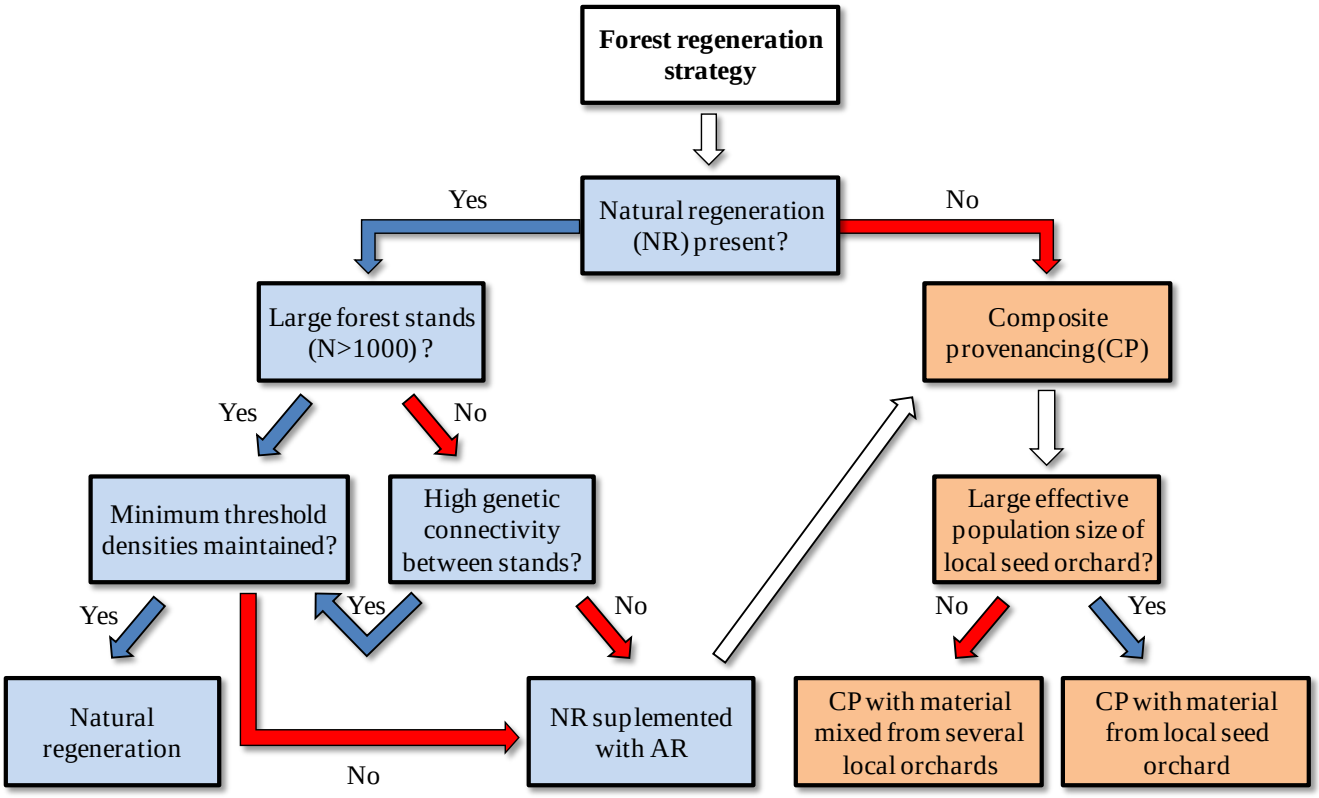


Figure 6.3. Regeneration strategy decision tree. Decisions strongly depend on the characteristics of the forest stands where the forest reproductive material is produced. AR: artificial regeneration, NR: natural regeneration, CP: composite provenancing

6.3 Research perspectives

In this study we tried to develop a general framework to assess the long term sustainability of fragmented forests in terms of tree genetic diversity. However, making generalizations regarding the negative genetic effects of fragmentation on tree populations is difficult, as their response may vary widely through differences in life history traits (mating system, pollination and dispersal biology), ecological and habitat specific factors. This highlights the necessity of a better understanding of a species history and ecology, before one can grasp or even predict the genetic consequences of forest fragmentation (Petit & Hampe 2006; Montoya et al. 2008). Moreover, since only a limited number of populations and individuals were used in

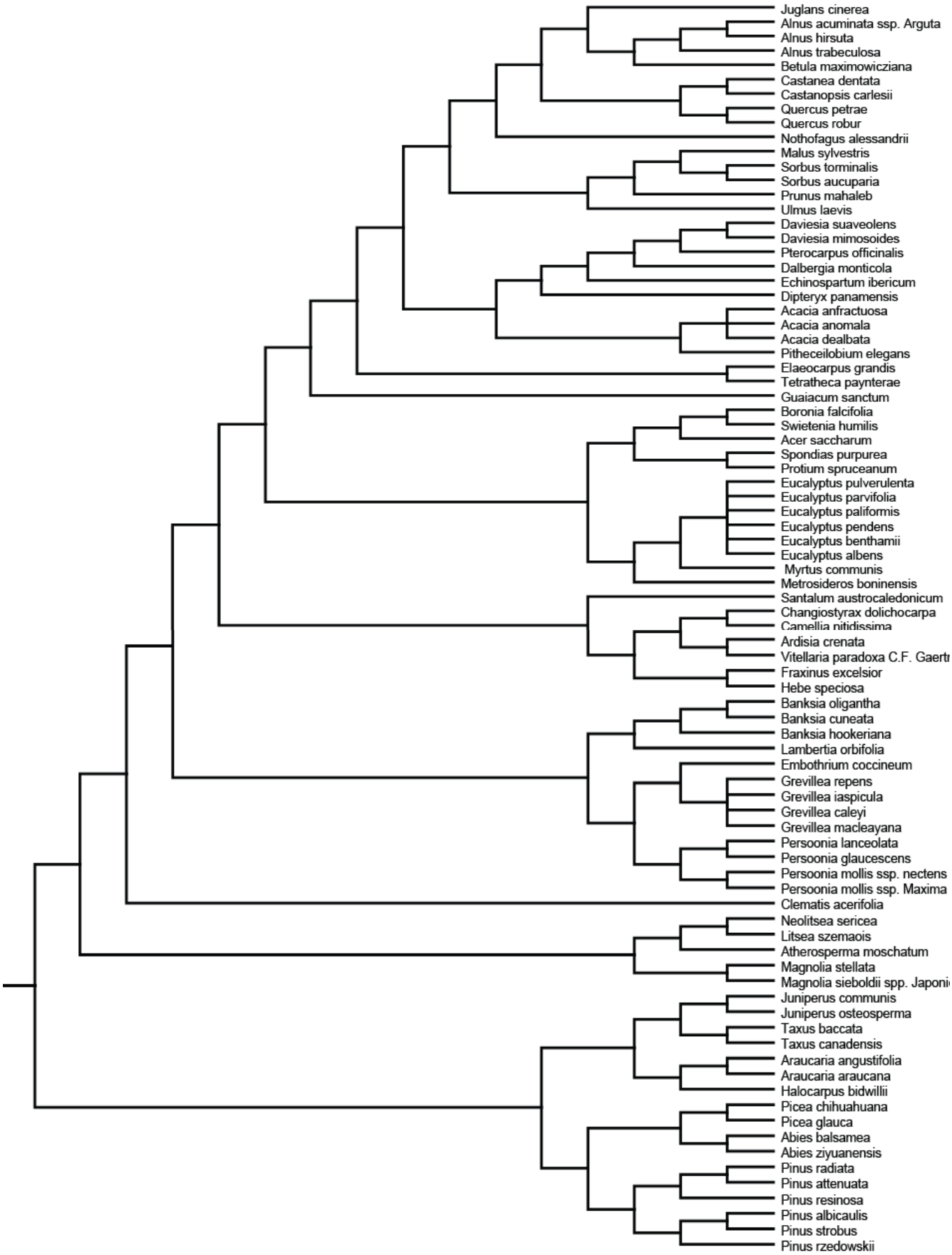
our study, some of the stand characteristics (isolation, density and landscape matrix) were confounded with each other. To disentangle these confounding effects and to allow rigorous statistical testing (parametric tests and general linear models) of the relationships between stand characteristics and mating and pollen flow patterns, more forest stands differing in population size, tree density, isolation and matrix type should be examined. Based on these additional stands one could try to establish a model for predicting the effects of anthropogenic disturbances or heterogeneity in the landscape on contemporary geneflow in pedunculate oak. Understanding genetic connectivity at the landscape scale is important for predicting the impacts of landscape changes on tree regeneration and evolutionary processes (Finkeldey & Ziehe 2004).

If geneflow is restricted between small and spatially isolated populations, conservation resources could be directed towards management practices that maintain or increase N_e in small-scale forestry systems. Although supplementation of natural regeneration by sowing or planting may theoretically increase N_e in small stands, little empirical understanding of how to select forest reproductive material to meet this challenge is available. Especially in view of climate change, assisted migration through combining local and more distant seed sources (composite provenancing) looks a promising regeneration method to maximize the evolutionary potential in small and fragmented forest stands. However, more information regarding the risk for outbreeding depression in artificial regenerated forest stands is necessary as mixed evidence for outbreeding depression exists in the scientific literature ranging from heterosis to poor progeny fitness (Edmands 2007). By sowing or planting different proportions of non-local reproductive material, collected at various distances from a study site (1 to several 100 kilometres), better guidelines for composite provenancing might be available in the future.

Also the integration of genomic techniques in forest landscape genetics will further increase knowledge in this field. Compared to neutral molecular markers like microsatellites and ALFPs, next-generation sequencing (NGS) have several advantages. First, studies using NGS are able to examine much more markers (single

nucleotide polymorphism (SNP)) than microsatellite studies (several 10,000 versus 10 - 100 markers), which allow the study of genome-wide genetic diversity (Ouborg et al. 2010). Second, contrary to microsatellites which are assumed to be selectively neutral, SNPs can be used to study the variation in functional genes, and hence may give insight in processes such as local adaptation or the evolutionary potential of species to respond to environmental changes (Allendorf et al. 2010). For example in our HFC study only the presence or absence of significant fitness responses to genetic erosion could be registered. Although more SNPs are needed to approximate the power of microsatellite loci and data interpretation will be more difficult with more loci, a better understanding of the genetic architecture in fragmented populations may become possible by using large sets of SNPs in HFC studies (Szulkin et al. 2010). This will provide increased insight into the relationship between genetic diversity and fitness and how they interact with environmental change. Such environmental interactions may have a major impact on the genetic consequences of anthropogenic disturbances in forests. Ecological factors that shape a species habitat (e.g., soil composition, light availability, competition with surrounding vegetation, etc.) may induce stronger effects on fitness than heterozygosity (Oostermeijer et al. 1994). Therefore predicting the exact response of tree species to global change will require a thorough understanding of these effects.

Appendices



Appendix 2.1. phylogenetic tree used in the present study to test the presence of a phylogenetic signal using the Phylometa 1.0 beta software (Lajeunesse 2009)

Appendix 2.2. Woody species and studies used for the meta-analysis on the relationship between genetic diversity and population size. For each record we obtained: the number of populations sampled in a study, the molecular marker used, the mating system, pollination vector, seed dispersal vector, longevity and studied tissue.

			Moderator variables					
Species	Study	*n*	marker	Mating system	Pollination vector	seed dispersal vector	Longevity (years)	Studied tissue
Abies balsamea	Shea & Furnier 2002	4	alloz	SC/MO	wind	wind	<100	adult
Abies ziyuanensis	Tang et al. 2008	7	SSR	no data	wind	wind	>100	adult
Acacia anfractuosa	Coates et al. 2006	6	alloz	SC	insect	ants	no data	progeny
Acacia anomala	Coates 1988	10	alloz	SC/MO	insect	ants	no data	adult
Acacia dealbata	Broadhurst et al. 2008	12	alloz	OO	insect	ants	<100	progeny
Acer saccharum	Young et al. 1993	8	alloz	SC/MO	wind	wind	>100	progeny
Acer saccharum	Baucom et al. 2005	16	alloz	SC/MO	wind	wind	>100	adult
Alnus acuminata	Murillo & Finkeldey 2000	17	alloz	OO	wind	wind	>100	adult
Alnus hirsuta	Huh & Huh 1999	15	alloz	OO	wind	wind	<100	adult
Alnus trabeculosa	Miyamoto et al. 2001	5	alloz	OO	wind	wind	<100	adult
Araucaria angustifolia	Auler et al. 2002	9	alloz	OO	wind	birds & mammals	>100	adult
Araucaria araucana	Bekessy et. al. 2002	13	RAPD	OO	wind	birds & mammals	>100	adult

Araucaria columnaris	Kettle et al. 2007	6	SSR	SC/MO	wind	wind	>100	progeny
Araucaria nemorosa	Kettle et al. 2007	6	SSR	SC/MO	wind	wind	>100	progeny
Ardisia crenata	Zhao et al. 2006	16	RAPD	SC/MO	insect	birds	<100	adult
Atherosperma moschatum	Shapcott 1994	22	alloz	SC/MO	insect	wind	>100	adult
Banksia cuneata	Broadhurst & Coates 2004	7	alloz	SC	bird	wind	<100	progeny
Banksia hookeriana	Krauss et al. 2006	15	AFLP	OO	bird	wind	<100	adult
Banksia oligantha	Broadhurst & Coates 2004	7	alloz	SC	bird	wind	<100	progeny
Betula maximowicziana	Uchiyama et al. 2006	6	SSR	OO	wind	wind	>100	adult
Boronia falcifolia	Shapcott et al. 2010	8	SSR	SC	insect	ejection	<100	adult
Camellia nitidissima	Xiao et al. 2008	13	SSR	no data	insect	gravity	<100	adult
Caryocar brasiliense	Collevatti et al. 2001	10	SSR	OO	bat	gravity	>100	adult
Castanea dentata	Huang et al. 1998	12	alloz	OO	insect	birds & mammals	>100	adult
Castanopsis carlesii	Cheng et al. 2006	22	alloz	OO	wind	gravity	no data	adult
Changiostyrax dolichocarpa	Yao et al. 2007	5	SSR	SC/MO	insect	gravity	<100	adult
Clematis acerifolia	López-Pujol et al. 2005	9	alloz	no data	no data	gravity	<100	adult
Dalbergia monticola	Olivarimbola et al. 2006	10	RAPD	SC	insect	gravity	>100	adult

Dalbergia monticola	Andrianoelina et al. 2009	18	SSR	SC	insect	gravity	>100	adult
Daviesia mimosoides	Young & Brown 1996	5	alloz	OO	insect	ants	<100	progeny
Daviesia suaveolens	Young & Brown 1996	5	alloz	OO	insect	ants	<100	progeny
Dipteryx panamensis	Hanson et al. 2008	8	SSR	SC	insect	mammals	>100	adult
Echinospartum ibericum	Aparicio et al. 2002	5	alloz	no data	insect	ejection	<100	progeny
Elaeocarpus grandis	Rossetto et al. 2004	21	SSR	no data	insect	birds & mammals	>100	both
Embothrium coccineum	Mathiasen et al. 2007	6	alloz	SC/MO	bird	gravity	no data	progeny
Eucalyptus albens	Prober & Brown 1994	25	alloz	SC/MO	insect	bats	no data	progeny
Eucalyptus benthamii	Butcher et al. 2005	4	SSR	SC	insect	gravity	>100	adult
Eucalyptus paliformis	Prober et al. 1990	6	alloz	no data	insect	gravity	no data	progeny
Eucalyptus parvifolia	Prober et al. 1990	8	alloz	no data	insect	gravity	no data	progeny
Eucalyptus pulverulenta	Peters et al. 1990	4	alloz	no data	insect	gravity	<100	progeny
Fagus sylvatica	Jump & Peñuelas 2006	14	SSR	OO	wind	birds & mammals	>100	progeny
Fraxinus excelsior	Bacles et al. 2005	5	SSR	SC	wind	wind	>100	adult
Grevillea caleyi	Llorens et al. 2004	8	AFLP	SC	bird	gravity	<100	adult
Grevillea iaspicula	Hoebee & Young 2001	5	alloz	OO	bird	gravity	<100	progeny

Grevillea macleayana	England et al. 2002	6	SSR	SC	bird	gravity	<100	adult
Grevillea repens	Holmes et al. 2009	6	SSR	OO	bird	no data	no data	adult
Guaiacum sanctum	Fuchs & Hamrick 2010	7	alloz	SC	insect	birds	>100	adult
Halocarpus bidwillii	Billington 1991	17	alloz	OO	wind	birds	>100	progeny
Hebe speciosa	Armstrong & De Lange FLS 2005	6	AFLP	OO	insect	gravity	no data	adult
Juglans cinerea	Ross-Davis et al. 2008	5	SSR	OO	wind	mammals	<100	adult
Juniperus communis	Oostermeijer & De Knegt 2004	12	alloz	OO	wind	birds & mammals	>100	adult
Juniperus communis	Michalczyk et al. 2008	8	SSR	OO	wind	birds & mammals	>100	adult
Juniperus osteosperma	Allphin et al.2007	5	alloz	OO	wind	mammals	>100	adult
Lambertia orbifolia	Coates & Hamley 1999	7	alloz	SC	bird	no data	no data	progeny
Litsea szemaois	Ci et al. 2008	8	AFLP	OO	insect	gravity	no data	adult
Magnolia sieboldii	Kikuchi & Isagi 2004	14	SSR	no data	insect	birds	no data	adult
Magnolia stellata	Tamaki et al. 2008	20	SSR	SC	insect	birds	>100	adult
Malus sylvestris	Larsen et al. 2006	4	SSR	OO	insect	mammals	<100	adult
Medicago citrina	Juan et al. 2004	9	SSR	SC	insect	no data	no data	adult
Metrosideros boninensis	Kaneko et al. 2008	4	SSR	no data	bird	wind	no data	adult

Metrosideros excelsa	Schmidt-Adam et al. 2000	5	alloz	SC	bird	wind	>100	progeny
Myrtus communis	González-Varo et al. 2010	6	alloz	SC	insect	birds	no data	progeny
Myrtus communis	Albaladejo et al. 2008	14	alloz	SC	insect	birds	no data	adult
Neolitsea sericea	Wang et al. 2005	6	RAPD	OO	insect	birds	>100	adult
Nothofagus alessandrii	Torres-Díaz et al. 2007	7	alloz	OO	wind	wind	>100	adult
Persoonia glaucescens	Rymer & Ayre 2006	4	AFLP	OO	insect	birds & mammals	no data	adult
Persoonia lanceolata	Rymer & Ayre 2006	4	AFLP	OO	insect	birds & mammals	no data	adult
Persoonia mollis maxima	Rymer & Ayre 2006	4	AFLP	OO	insect	birds & mammals	<100	adult
Persoonia mollis nectens	Rymer & Ayre 2006	4	AFLP	OO	insect	birds & mammals	<100	adult
Picea chihuahuana	Ledig et al. 1997	7	alloz	SC	wind	no data	>100	progeny
Picea glauca	O'Connell et al. 2006	23	alloz	SC	wind	wind	>100	progeny
Pinus albicaulis	Krakowski et al. 2003	13	alloz	SC	wind	birds	>100	adult
Pinus pinaster	De-Lucas et al. 2009	4	SSR	SC	wind	wind	>100	adult
Pinus radiata	Karhu et al. 2005	5	SSR	no data	wind	birds & mammals	>100	progeny
Pinus resinosa	Boys et al. 2005	17	SSR	SC	wind	wind	>100	adult
Pinus rzedowskii	Delgado et al. 1999	9	alloz	no data	wind	wind	>100	both

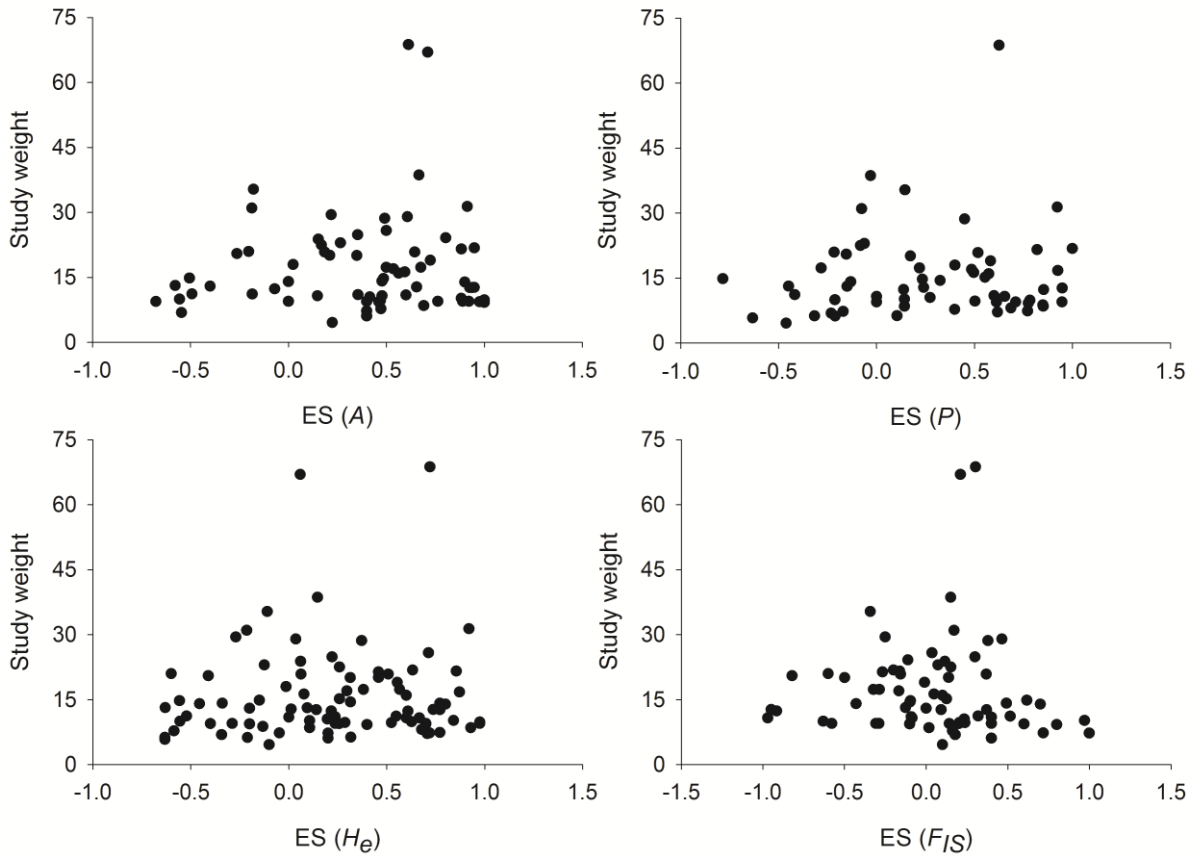
Pinus strobus	Buchert et al. 1997	4	alloz	SC	wind	wind	>100	progeny
Pinus strobus	Marquardt et al. 2007	6	SSR	SC	wind	wind	>100	adult
Pithecellobium elegans	Hall et al. 1995	8	alloz	OO	insect	birds	>100	both
Plathymenia reticulata	Lacerda et al. 2001	6	RAPD	SC/MO	insect	wind	>100	adult
Protium spruceanum	Almeida Vieira & de Carvalho 2008	5	alloz	OO	insect	birds	>100	adult
Prunus africana	Farwig et al. 2008	8	SSR	OO	insect	birds & mammals	>100	progeny
Prunus mahaleb	Jordano & Godoy 2000	7	RAPD	SC/MO	insect	birds & mammals	>100	adult
Pterocarpus officinalis	Muller et al. 2009	7	SSR	no data	insect	water	no data	adult
Quercus humboldtii	Fernández-M & Sork 2005	6	SSR	SC	insect	birds & mammals	>100	adult
Quercus petraea	Mariette et al. 2001	7	SSR	SC/MO	wind	birds & mammals	>100	adult
Quercus robur	Vakkari et al. 2006	33	alloz	SC/MO	wind	birds & mammals	>100	adult
Quercus robur	Mariette et al. 2001	7	SSR	SC/MO	wind	birds & mammals	>100	both
Santalum austrocaledonicum	Bottin et al. 2005	5	SSR	SC	insect	birds	>100	adult
Sorbus aucuparia	Raspé & Jacquemart 1998	17	alloz	OO	insect	birds & mammals	>100	adult
Sorbus aucuparia	Bacles et al. 2005	8	alloz	OO	insect	birds & mammals	>100	adult
Sorbus torminalis	Hoebee et al. 2006	10	alloz	OO	insect	birds & mammals	>100	adult

Sorbus torminalis	Rasmussen & Kollmann 2008	6	SSR	OO	insect	birds & mammals	>100	both
Spondias purpurea	Miller & Schaal 2006	13	AFLP	OO	insect	animals	no data	adult
Swietenia humilis	White et al. 1999	4	SSR	OO	insect	wind	>100	adult
Swietenia macrophylla	Novicket al. 2003	8	SSR	SC	insect	gravity	>100	adult
Symphonia globulifera	Aldrich et al. 1998	6	SSR	SC	bird	birds & mammals	no data	progeny
Tabebuia ochracea	Moreira et al. 2009	4	SSR	OO	insect	wind	>100	progeny
Taxus baccata	Myking et al. 2009	13	alloz	OO	wind	bird	>100	adult
Taxus baccata	Dubreuil et al. 2010	4	SSR	OO	wind	bird	>100	adult
Taxus canadensis	Senneville et al. 2000	6	alloz	no data	wind	birds & mammals	>100	progeny
Terminalia amazonia	Pither et al. 2003	6	RAPD	no data	insect	wind	>100	both
Tetratheca paynterae	Butcher et al. 2009	5	SSR	no data	insect	ants	no data	adult
Ulmus laevis	Vakkari et al. 2009	13	alloz	OO	wind	wind	>100	adult
Vitellaria paradoxa	Fontaine et al. 2004	13	RAPD	SC/MO	insect	birds & mammals	>100	adult

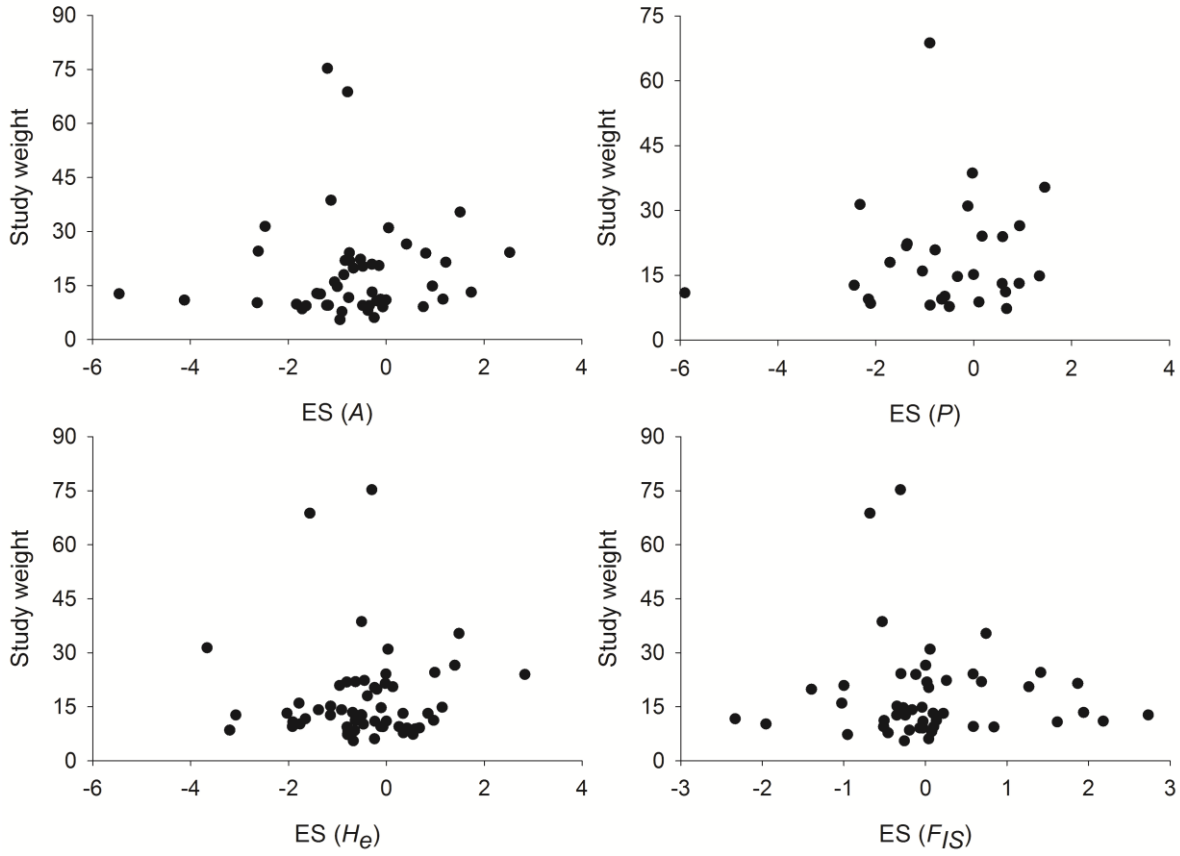
n*: number of populations; Genetic marker: alloz: allozymes, SSR: microsatellites, RAPD, AFLP; Mating system: SC: self-compatible, OO: obligate outcrossing, SC/MO: self-compatible but mainly outcrossing; Pollination: wind, insect or bird pollination; Seed dispersal vector: wind, gravity, birds or mammals; longevity: less or more than 100 years; Studied tissue: adult, progeny or both*.

Appendix 2.3. The number of data points associated with the Spearman rank correlation coefficient, Hedge's *d* and both types of effect sizes. (*A*: number of alleles per locus; *P*: percentage polymorphic loci; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient).

	Number of data points		
Genetic diversity measure	Spearman rank correlation	Hedge's *d*	Both effect sizes
A	68	49	38
P	61	29	26
H_e	89	59	44
F_{IS}	71	49	36



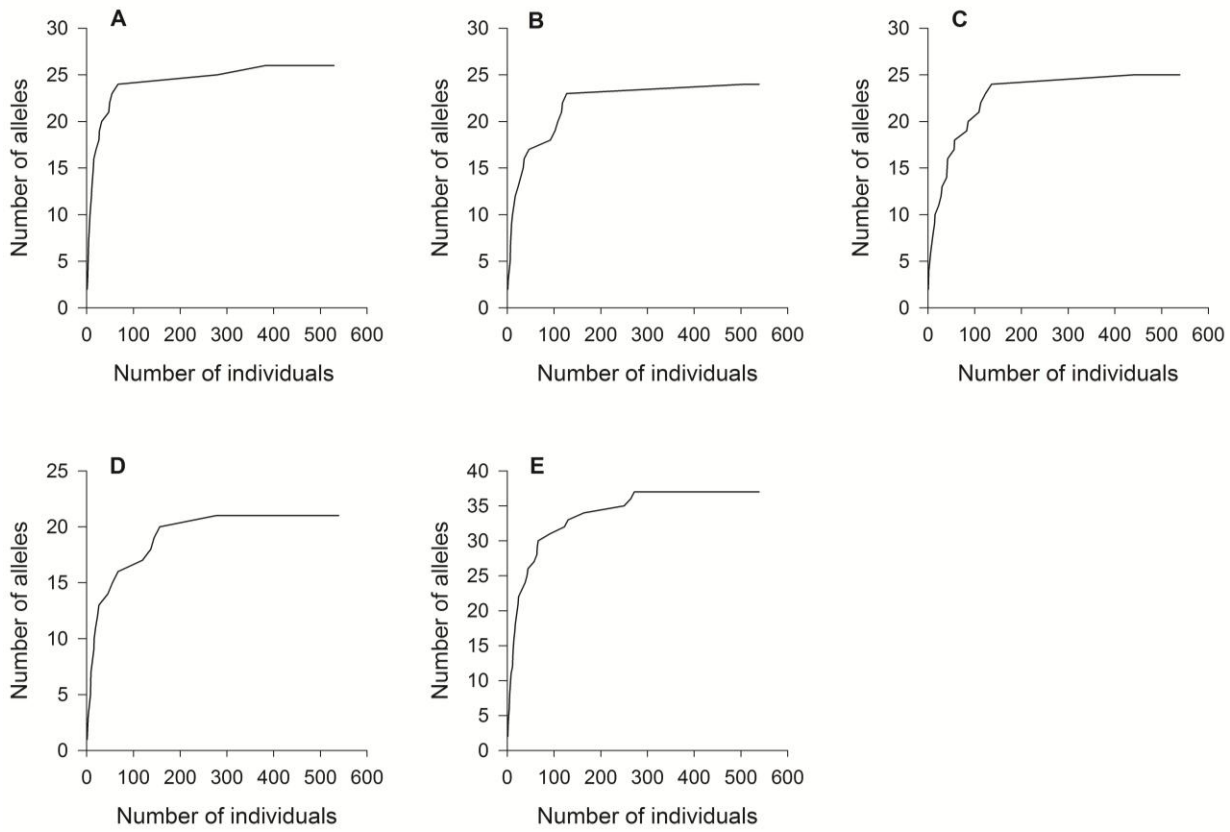
Appendix 2.4. Funnel plots of effect size (ES) using the Spearman rank correlation coefficient versus study weight for four genetic diversity measures. (*A*: number of alleles per locus; *P*: percentage polymorphic loci; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient).



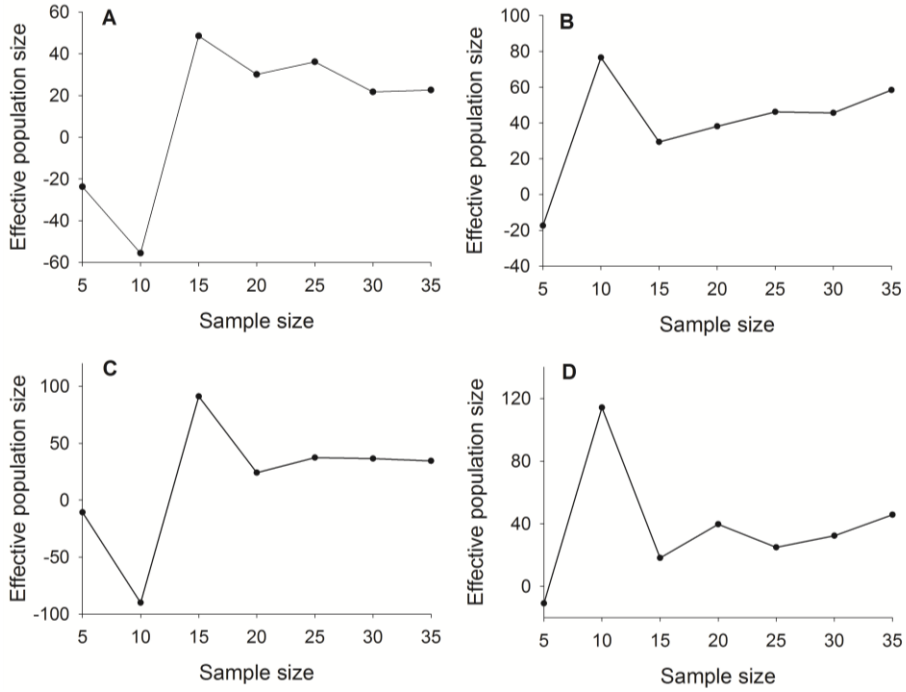
Appendix 2.5. Funnel plots of effect size (ES) using Hedge's *d* versus study weight for four genetic diversity measures. (*A*: number of alleles per locus; *P*: percentage polymorphic loci; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient).

Appendix 3.1. Null allele frequencies for the 10 SSR loci in the 4 studied forest stands measured with Genepop v.4 (Rousset 2008).

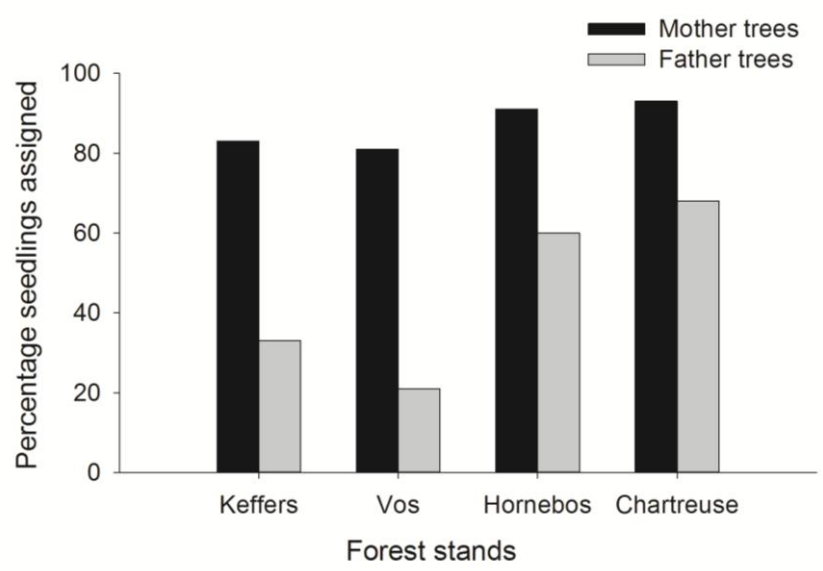
	Keffers	Chartreuse	Hornebos	Vos
QpZAG9	0.0178	0	0	0
MSQ14	0.0316	0.0235	0.0035	0.0407
QpZAG46	0.0338	0	0.0037	0.0312
QpZAG15	0	0	0	0.0012
QpZAG110	0	0.0035	0.0047	0
MSQ13	0	0	0	0
MSQ16	0.183	0.1155	0.169	0.1277
QrZAG112	0	0	0	0
QpZAG108	0.0738	0.0799	0.053	0.0496
QpZAG104	0	0.0005	0	0



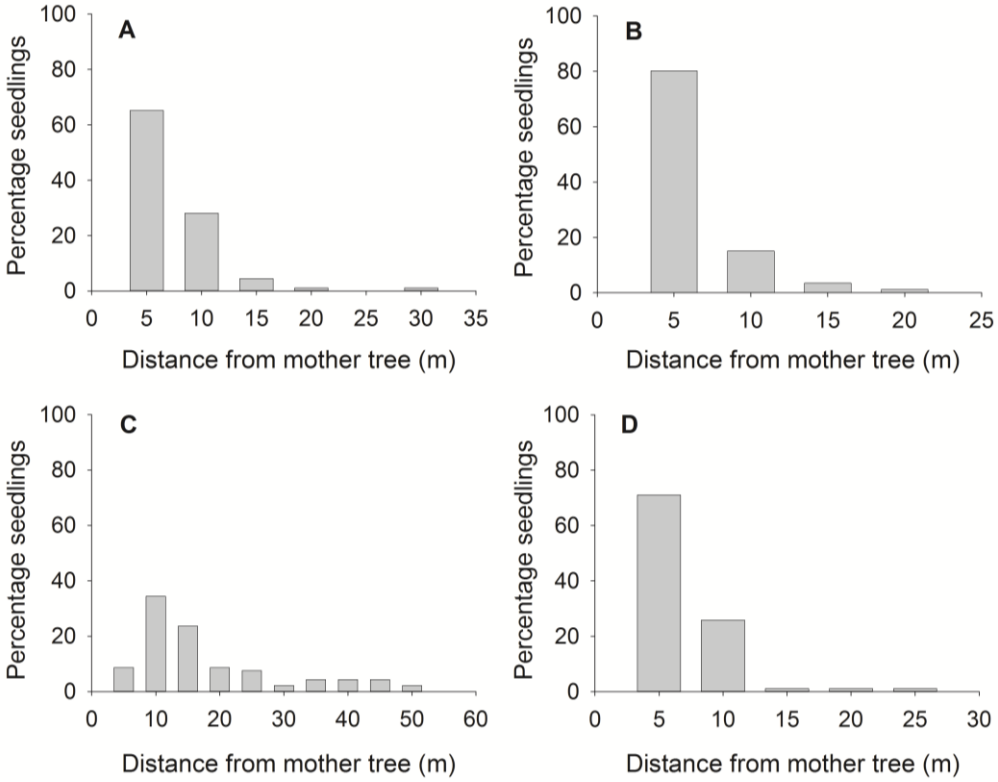
Appendix 3.2. The number of alleles plotted against the number of individuals, for microsatellites with a high number of alleles (> 20 alleles); QpZAG108 (A), QpZAG46 (B), QrZAG112 (C), QpZAG110 (D), QpZAG104 (E).



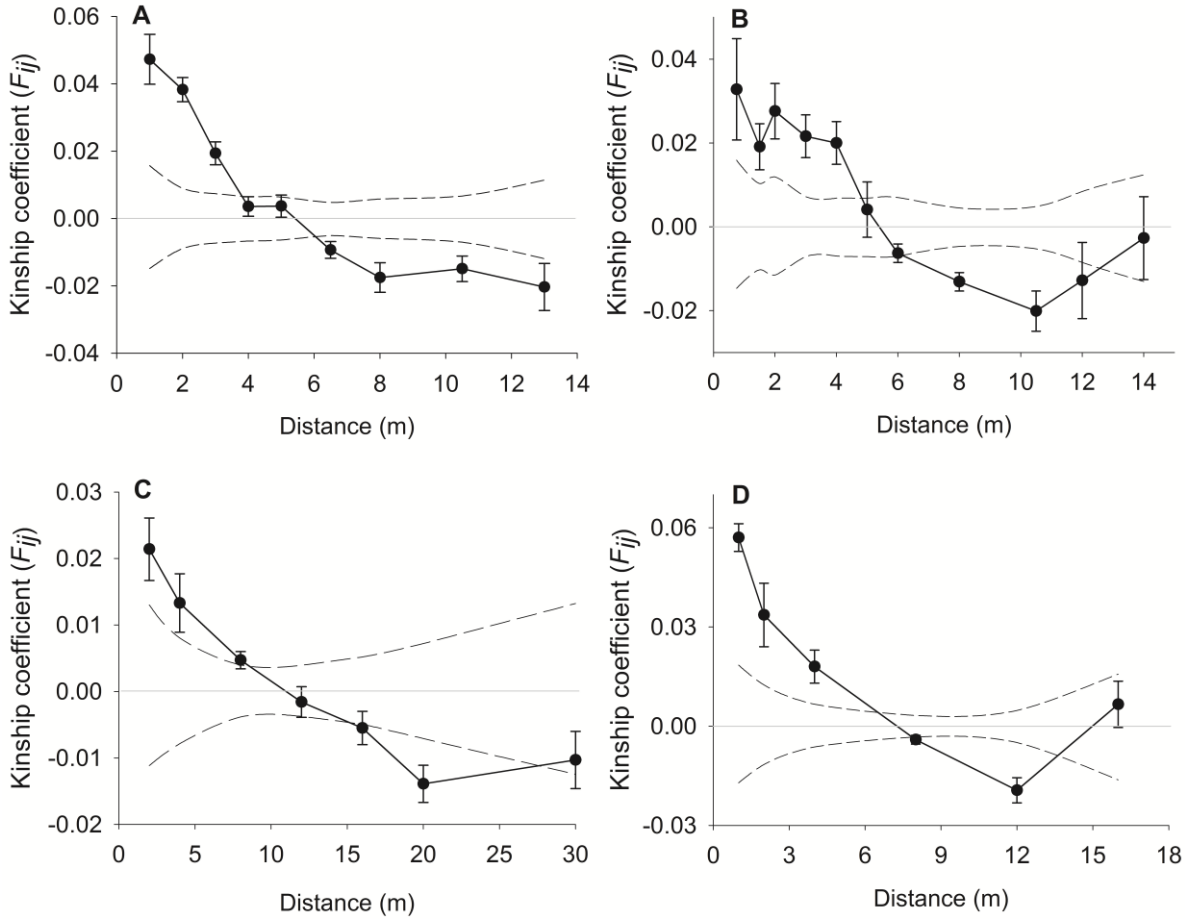
Appendix 3.3. Estimates of effective population size (N_e) plotted against sample size (5 - 35 samples) for the four studied forest stands, Chartreuse (A), Vos (B), Hornebos (C), Keffers (D).



Appendix 3.4. Percentages seedlings that were assigned to a mother and father tree from within the study plot.



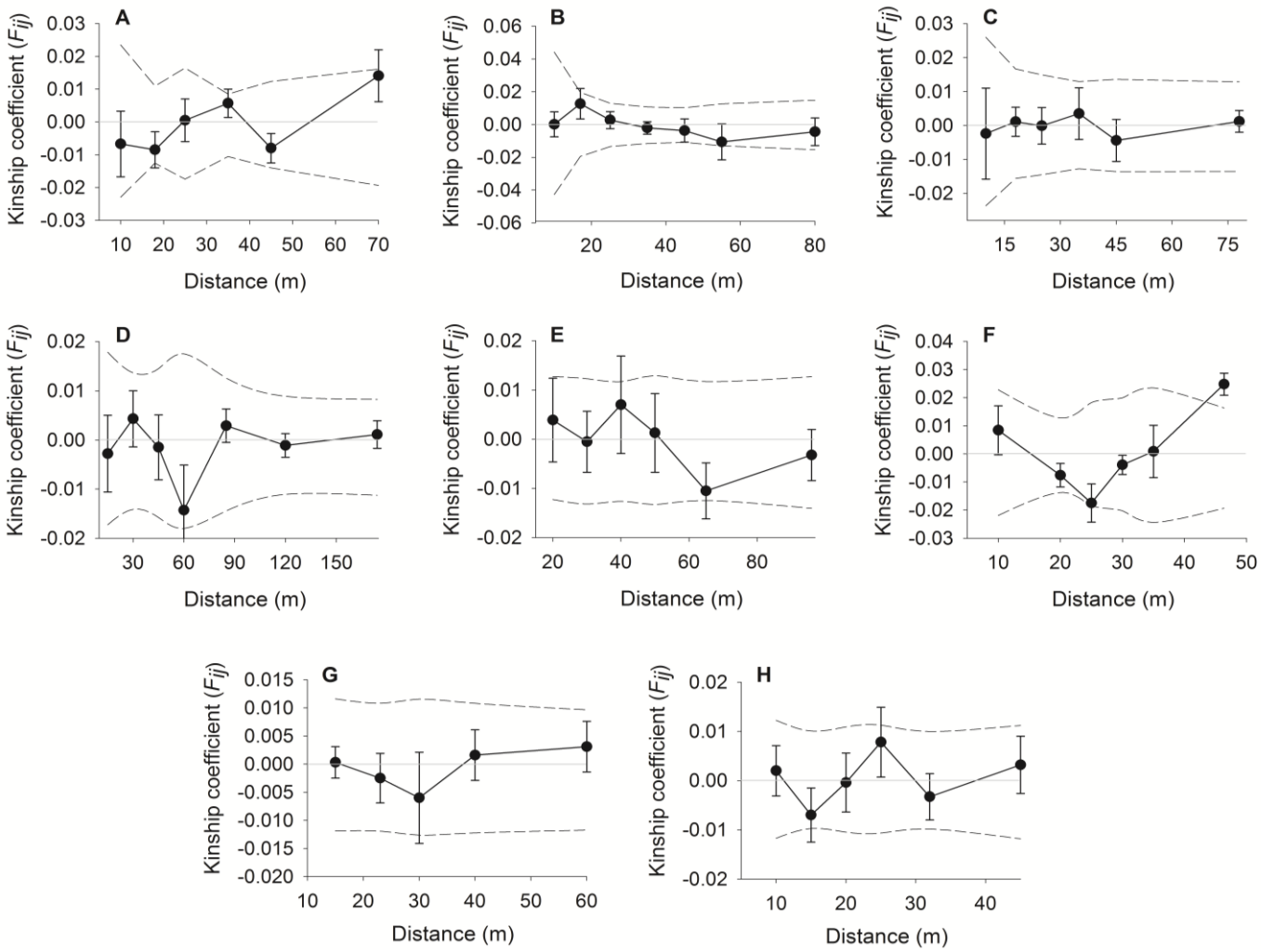
Appendix 3.5. Seed dispersal distributions inferred from the parentage analysis in four forest stands: Keffers (A), Vos (B), Hornebos (C), Chartreuse (D).



Appendix 3.6. Auto-correlograms of Nason's kinship coefficients (F_{ij}) for seedling pairs in four forest stands: Keffers (A), Vos (B), Hornebos (C), Chartreuse (D). The dashed lines indicate upper and lower 95% confidence intervals (10,000 permutations).

Appendix 4.1. Null allele frequencies for the 10 microsatellite loci in the 8 studied forest stands measured with Genepop v.4 (Raymond & Rousset 1995)

Forest stand	*QpZAG9*	*MSQ14*	*QpZAG46*	*QpZAG15*	*QpZAG110*	*MSQ13*	*MSQ16*	*QrZAG112*	*QpZAG108*	*QpZAG104*
Keffers	0.01	0.047	0.077	0.021	0	0	0.146	0	0.0129	0
Vos	0	0.053	0.06	0.015	0	0.038	0.088	0	0.045	0
Hornebos	0	0	0	0	0	0	0.073	0	0.045	0
Chartreuse	0.011	0	0.021	0	0	0	0.137	0	0.01	0.026
Overheide	0	0.028	0.006	0	0	0	0.046	0	0.044	0
Hoge Vijvers	0.011	0	0	0.043	0	0	0.05	0.032	0.056	0.02
Meikensbos	0	0.013	0.064	0.043	0.019	0.033	0.062	0	0.032	0.011
Egemse Veldekens	0	0	0.025	0	0	0.024	0.108	0	0	0



Appendix 4.2. Nason's kinship coefficients (F_{ij}) ± standard errors for adult pairs in the eight studied forest stands: (A) Overheide, (B) Meikensbos, (C) Chartreusebos, (D) Egemse Veldekens, (E) Hornebos, (F) Hoge Vijvers, (G) Keffers, (H) Vos. The dashed lines indicate upper and lower 95% confidence intervals (10,000 permutations).

Appendix 5.1. Results of the Ewens-Watterson homozygosity test of neutrality (Manly 1985). The 95% lower and upper confidence limits (L95* and U95*) and standard errors for the observed *F* (sum of square of allele frequencies) were calculated for all microsatellites using 100,000 simulated samples in Popgene version 1.32. For all loci the observed *F* value falls within the 95% confidence interval.

Locus	*n*	*k*	Obs. *F*	SE	L95*	U95*
MSQ14	147	17	0.116	0.005	0.113	0.399
QpZAG108	147	25	0.086	0.002	0.080	0.250
QpZAG9	148	14	0.154	0.009	0.133	0.494
QpZAG110	147	14	0.288	0.009	0.133	0.494
QpZAG15	147	10	0.295	0.016	0.174	0.660
QpZAG46	147	22	0.239	0.003	0.090	0.297
MSQ13	148	11	0.199	0.013	0.161	0.615
QpZAG104	147	25	0.081	0.002	0.080	0.252
QrZAG112	147	21	0.152	0.003	0.094	0.314

Abbreviations: n*: number of samples;* k*: No. of alleles; SE: Standard error for the observed* F

Appendix 5.2. Results of the F_{st} outlier test implemented in Arlequin v.3.5. (Excoffier & Lischer 2010). Observed levels of heterozygosity, F_{st} and *p*-values for F_{st} were calculated for all microsatellites.

Locus	Observed Heterozygosity	Observed F_{st}	F_{st} *p*-value
MSQ14	0.896	0.030	0.469
QpZAG108	0.925	0.025	0.312
QpZAG9	0.857	0.030	0.430
QpZAG110	0.726	0.048	0.235
QpZAG15	0.713	0.021	0.277
QpZAG46	0.774	0.044	0.275
MSQ13	0.814	0.037	0.313
MSQ16	0.795	0.032	0.414
QpZAG104	0.931	0.029	0.405
QrZAG112	0.856	0.029	0.417

Appendix 5.3. Linear regression models relating biomass data to morphological input variables based on 50 harvested seedlings. Estimates for each forest stand were used for the calculation of initial biomass of the 100 other seedlings.

Dependent variable	Forest stand	F_{model}	R^2_{adj}[a]	Mallow's C_p	Constant	Ln(V_{tot})	Ln(S_L)	Ln (L_n)	B_n
Total dry initial biomass	Keffers	154.047***	0.91	2.0	1.401***	0.562***	/	/	/
	Vos	24.647***	0.84	4.0	2.681***	0.715***	-0.600**	0.209(*)	/
	Chartreuse	133.258***	0.95	3.0	3.371***	1.041***	-0.840***	/	/
Total fresh initial biomass	Keffers	84.056***	0.91	3.0	2.846***	0.673***	-0.172	/	/
	Vos	37.018***	0.84	3.0	2.564***	0.433***	/	0.112	/
	Chartreuse	50.458***	0.93	5.0	3.973***	0.967***	-0.856***	0.249	-0.081
Dry aboveground biomass	Keffers	147.128***	0.95	3.0	-0.420(*)	0.841***	/	0.124	/
	Vos	28.095***	0.95	3.0	-0.261	0.549***	/	0.288**	/
	Chartreuse	120.555***	0.97	5.0	0.602	0.940***	-0.607**	0.445**	-0.083

[a] *The models with the highest* R^2_{adj} *were selected for initial biomass estimation*

Abbreviations: V_{tot}*, Total woody volume;* S_L*, stem length;* L_n*, number of leaves;* B_n*, number of branches*

*(*) 0.05 ≤ p < 0.1; ** 0.001 ≤ p < 0.05; *** p < 0.001*

Appendix 5.4. Results of the initial linear regression models (before model reduction) performed to examine the effect of irrigation regime, MLH and their interaction on (A) transpiration and (B) growth trait variables for *Q. robur*.

		Correlation model		Irrigation		MLH		Irrigation x MLH		Forest stand		Log seed weight	
Parameter	Variable	F_{model}	R^2_{adj}	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p
(A) Transpiration													
Stomatal conductance	log g_s day35 a.m.	35.48***	0.68	10.16***	0.10	4.67**	0.05	0.36	< 0.01	4.52**	0.09	< 0.01	< 0.01
	log g_s day35 p.m.	37.34***	0.69	10.42**	0.10	5.67**	0.06	0.31	< 0.01	1.45	0.03	0.03	< 0.01
	log g_s day49 p.m.	92.28***	0.85	35.54***	0.28	8.21**	0.08	3.20(*)	0.03	5.74**	0.11	0.80	< 0.01
	log g_s day65 p.m.	100.56***	0.86	16.29***	0.15	10.57**	0.10	0.17	< 0.01	2.75*	0.06	0.75	< 0.01
Leaf water potential	ψ_{md}	20.93***	0.68	14.92***	0.23	2.13	0.04	2.27	0.04	1.10	0.04	2.04	0.04
	ψ_{pd}	30.85***	0.75	6.29**	0.76	< 0.01	< 0.01	0.30	< 0.01	0.93	0.03	2.13	0.04
Water potential range	$\Delta\psi$	5.23***	0.31	8.60**	0.15	2.53	0.05	4.16	0.08	0.75	0.03	2.28	0.04
Total transpiration$_{corr}$	TR	58.66***	0.79	3.56(*)	0.04	1.34	0.02	2.54	0.03	2.39 (*)	< 0.05	1.64	0.02
Water content	WC	30.03***	0.65	5.86**	0.06	3.66(*)	0.04	< 0.01	< 0.01	0.19	< 0.01	0.13	< 0.01
(B) Growth traits													
RGR (Evans 1972)	RGR$_{diameter}$	45.31***	0.74	19.71***	0.18	3.37(*)	0.04	2.15	0.02	0.96	< 0.01	2.62	0.03
	RGR$_{length}$	2.46**	0.08	0.02	< 0.01	2.47	0.03	0.42	< 0.01	1.66	0.04	1.14	0.01
	RGR$_{woody\ volume}$	30.58***	0.65	14.92***	0.14	4.36**	0.05	2.14	0.02	0.41	< 0.01	2.65	0.03
	RGR$_{dry\ biomass}$	32.99***	0.67	7.35**	0.08	1.80	0.02	0.02	< 0.01	0.61	0.01	1.47	0.02
	RGR$_{fresh\ biomass}$	50.99***	0.76	14.09***	0.14	1.98	0.02	0.29	< 0.01	0.07	< 0.01	2.04	0.02
	RGR$_{aboveground}$	15.07***	0.47	8.38**	0.09	2.36	0.03	1.74	0.02	5.14**	0.10	0.04	< 0.01
	RGR$_{roots}$	20.80***	0.56	2.93(*)	0.03	0.12	< 0.01	0.05	< 0.01	6.55**	0.13	4.03**	0.043
Transpiration efficiency	TE	1.69	0.04	0.50	< 0.01	0.01	< 0.01	0.43	< 0.01	0.33	< 0.01	7.60**	0.08

To account for the differences between forests and seed sizes, forest stand and log seed weight were included in the analysis as a fixed effect. F-*statistics,* R^2_p *coefficients and significance levels for main effects and interactions are presented for the initial models. (*) 0.05 ≤ p < 0.1; ** 0.001 ≤ p < 0.05; *** p < 0.001.*

Appendix 5.5. Results of the linear regression models after exclusion of the locus QpZAG104, to examine the effect of irrigation regime, MLH and their interaction on (A) transpiration and (B) growth trait variables for *Q. robur*.

		Correlation model		Irrigation		MLH		Irrigation x MLH		Forest stand		Log seed weight	
Parameter	Variable	F_{model}	R^2_{adj}	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p
(A) Transpiration													
Stomatal conductance	log g_s day35 a.m.	41.94***	0.68	10.94**	0.11	3.52(*)	0.04	0.20	< 0.01	4.41**	0.09		
	log g_s day35 p.m.	71.96***	0.68	12.50**	0.12	5.39**	0.05	0.38	< 0.01				
	log g_s day49 p.m.	108.49***	0.84	39.24***	0.29	8.23**	0.08	2.82(*)	0.03	5.21**	0.10		
	log g_s day65 p.m.	122.38***	0.86	17.99***	0.16	11.59***	0.11	0.47	< 0.01	2.66(*)	0.05		
Leaf water potential	ψ_{md}	40.91***	0.68	16.37***	0.24	3.35(*)	0.06	2.10	0.04				
	ψ_{pd}	58.94***	0.74	6.98**	0.11	0.06	< 0.01	0.37	< 0.01				
Water potential range	$\Delta\psi$	10.02***	0.33	10.05**	0.16	4.07**	0.07	4.54**	0.08				
Total transpiration$_{corr}$	TR	113.72***	0.78	3.79(*)	0.04	2.72	0.03	3.45(*)	0.04				
Water content	WC	62.41***	0.66	6.44**	0.07	5.17**	0.05	0.04	< 0.01				
(B) Growth traits													
RGR (Evans 1972)	$RGR_{diameter}$	69.40***	0.74	19.96***	0.18	4.9**	0.05	1.34	0.251			3.17(*)	0.03
	RGR_{length}	4.23***	0.09	0.20	< 0.01	4.31**	0.05	1.01	.01				
	$RGR_{woody\ volume}$	46.71***	0.66	14.82***	0.14	6.41**	0.07	1.41	0.02			2.73	0.03
	$RGR_{dry\ biomass}$	65.56***	0.67	7.64**	0.08	4.12(*)	0.04	0.04	< 0.01				
	$RGR_{fresh\ biomass}$	102.45***	0.76	14.41***	0.14	3.34(*)	0.04	0.05	< 0.01				
	$RGR_{aboveground}$	18.07***	0.47	7.49**	0.08	3.09(*)	0.03	0.98	0.01	5.39**	0.11		
	RGR_{roots}	20.84***	0.56	3.37(*)	0.04	0.18	< 0.01	0.10	< 0.01	6.55**	0.13	3.96(*)	0.04
Transpiration efficiency	TE	2.37(*)	0.06	0.40	< 0.01	0.03	< 0.01	0.33	< 0.01			8.36**	0.09

To account for the differences between forests and seed sizes, forest stand and log seed weight were included in the analysis as a fixed effect. F-*statistics,* R^2_p *coefficients and significance levels for main effects and interactions are presented for the final models. (*) 0.05 ≤ p < 0.1; ** 0.001 ≤ p < 0.05; *** p < 0.001*

Appendix 5.6. Results of the linear regression models after exclusion of the locus QpZAG46, to examine the effect of irrigation regime, MLH and their interaction on (A) transpiration and (B) growth trait variables for *Q. robur*.

		Correlation model		Irrigation		MLH		Irrigation x MLH		Forest stand		Log seed weight	
Parameter	Variable	F_{model}	R^2_{adj}	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p	F	R^2_p
(A) Transpiration													
Stomatal conductance	log g_s day35 a.m.	43.67***	0.69	7.88**	0.08	5.97**	0.06	0.12	< 0.01	5.67**	0.11		
	log g_s day35 p.m.	72.84***	0.70	5.12**	0.05	7.10**	0.07	0.05	< 0.01				
	log g_s day49 p.m.	108.75***	0.85	30.85***	0.25	7.56**	0.07	2.79(*)	0.03	6.31**	0.12		
	log g_s day65 p.m.	122.39***	0.86	13.27***	0.12	11.57***	0.11	0.31	< 0.01	3.55**	0.07		
Leaf water potential	ψ_{md}	40.15***	0.68	12.23***	0.19	2.69	0.05	1.62	0.03				
	ψ_{pd}	58.58***	0.76	5.91**	0.10	0.05	< 0.01	0.11	< 0.01				
Water potential range	$\Delta\psi$	9.03***	0.30	6.63**	0.11	3.09(*)	0.06	2.82(*)	0.05				
Total transpiration$_{corr}$	TR	108.95***	0.77	3.90(*)	0.04	1.33	0.01	1.47	0.02				
Water content	WC	60.70***	0.65	6.65**	0.07	3.00(*)	0.03	0.07	< 0.01				
(B) Growth traits													
RGR (Evans 1972)	$RGR_{diameter}$	88.41***	0.73	18.89***	0.17	1.32	0.01	2.39	0.03				
	RGR_{length}	3.78**	0.08	0.23	< 0.01	3.33(*)	0.04	0.90	0.01				
	$RGR_{woody\ volume}$	45.23***	0.65	14.08***	0.13	2.92(*)	0.03	2.23	0.02			2.03	0.02
	$RGR_{dry\ biomass}$	65.64***	0.67	7.04**	0.07	3.80(*)	0.04	0.06	< 0.01				
	$RGR_{fresh\ biomass}$	103.73***	0.76	15.26***	0.14	2.93(*)	0.03	0.74	< 0.01				
	$RGR_{aboveground}$	18.51***	0.51	9.39**	0.09	2.04	0.02	2.46	0.03	5.28**	0.11		
	RGR_{roots}	20.83***	0.58	2.92(*)	0.03	0.23	< 0.01	0.01	< 0.01	6.70**	0.13	3.91(*)	0.04
Transpiration efficiency	TE	2.53**	0.06	0.55	< 0.01	0.29	< 0.01	0.50	< 0.01			8.28**	0.08

To account for the differences between forests and seed sizes, forest stand and log seed weight were included in the analysis as a fixed effect. F-*statistics,* R^2_p *coefficients and significance levels for main effects and interactions are presented for the final models. (∗) 0.05 ≤* p *< 0.1; ∗∗ 0.001 ≤* p *< 0.05; ∗∗∗* p *< 0.001.*

References

References

Aas G. 1991. Crossing experiments with pedunculate and sessile oak (*Quercus robur and Q. petraea*). *Allgemeine Forst- Und Jagdzeitung* 162: 141-145.

Abrams MD. 2003. Where has all the white oak gone? *Bioscience* 53: 927-939.

Adams DC, Gurevitch J, Rosenberg MS. 1997. Resampling tests for meta-analysis of ecological data. *Ecology* 78: 1277-1283.

Aguilar R, Quesada M, Ashworth L, Herrerias-Diego Y, Lobo J. 2008. Genetic consequences of habitat fragmentation in plant populations: susceptible signals in plant traits and methodological approaches. *Molecular Ecology* 17: 5177-5188.

Alig RJ, Plantinga AJ, Ahn S, Kline JD. 2003. Land use changes involving forestry in the United States: 1952 to 1997, with projections to 2050. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, USA.

Allendorf FW, Hohenlohe PA, Luikart G. 2010. Genomics and the future of conservation genetics. *Nature Reviews Genetics* 11: 697-709.

Aravanopoulos FA, Zsuffa L. 1998. Heterozygosity and biomass production in *Salix eriocephala*. *Heredity* 81: 396-403.

Armbruster P, Reed DH. 2005. Inbreeding depression in benign and stressful environments. *Heredity* 95: 235-242.

Ashworth L, Aguilar R, Galetto L, Aizen MA. 2004. Why do pollination generalist and specialist plant species show similar reproductive susceptibility to habitat fragmentation? *Journal of Ecology* 92: 717-719.

Audo MC, Diehl WJ. 1995. Effect of quantity and quality of environmental stress on multilocus heterozygosity-growth relationships in *Eisenia fetida* (Annelida: Oligochaeta). *Heredity* 75: 98-105.

Austerlitz F, Smouse PE. 2001. Two-generation analysis of pollen flow across a landscape. III. Impact of adult population structure. *Genetic Research* 78(3): 271-280.

Bacles CFE, Lowe AJ, Ennos RA. 2004. Genetic effects of chronic habitat fragmentation on tree species: the case of *Sorbus aucuparia* in a deforested Scottish landscape. *Molecular Ecology* 13: 573-584.

Bacles CFE, Burczyk J, Lowe AJ, Ennos RA. 2005. Historical and contemporary mating patterns in remnant populations of the forest tree *Fraxinus excelsior* L. *Evolution* 59: 979-990.

Bacles CFE, Ennos RA. 2008. Paternity analysis of pollen mediated gene flow for *Fraxinus excelsior* L. in a chronically fragmented landscape. *Heredity* 101: 368-380.

Bacles CF, Jump AS. 2011. Taking a tree's perspective on forest fragmentation genetics. *Trends in Plant Science* 16(1): 13-18.

Balloux F, Amos W, Coulson T. 2004. Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology* 13(10): 3021-3031.

Barreneche T, Bodenes C, Lexer C, Trontin JF, Fluch S, Streiff R, Plomion C, Roussel G, Steinkellner H, Burg K, Favre J-M, Glössl J, Kremer A. 1998. A genetic linkage map of *Quercus robur* L. (pedunculate oak) based on RAPD, SCAR, microsatellite, minisatellite, isozyme and 5S rDNA markers. *Theoretical and Applied Genetics* 97(7): 1090-1103.

Barrett SCH, Kohn J. 1991. The genetic and evolutionary consequences of small population size in plant: implications for conservation. In: Falk D, Holsinger KE. (ed) *Genetics and conservation of rare plants.* Oxford University Press, Oxford, United Kingdom, pp 3-30.

Barrett RDH, Schluter D. 2008. Adaptation from standing genetic variation. *Trends in Ecology and Evolution* 23: 38-44.

Bary-Lenger A, Nebout JP. 1993. Le chêne. Editions du Perron, Alleur-Liège, Belgium.

Bauerle TL, Richards JH, Smart DR, Eissenstat DM. 2008. Importance of internal hydraulic redistribution for prolonging lifespan of roots in dry soil. *Plant Cell & Environment* 31: 177-186.

Bianchi FJ, Cunningham SA. 2012. Unravelling the role of mate density and sex ratio in competition for pollen. *Oikos* 121(2): 219-227.

Bijlsma R, Bundgaard J, Van Putten WF. 1999. Environmental dependence of inbreeding depression and purging in *Drosophila melanogaster*. *Journal of Evolutionary Biology* 12: 1125-1137.

Bittencourt JVM, Sebbenn AM. 2007. Patterns of pollen and seed dispersal in a small, fragmented population of the wind-pollinated tree *Araucaria angustifolia* in southern Brazil. *Heredity* 99(6): 580-591.

Bittencourt JVM, Sebbenn AM. 2008. Pollen movement within a continuous forest of wind-pollinated *Araucaria angustifolia*, inferred from paternity and TWOGENER analysis. *Conservation Genetics* 9(4): 855-868.

Bond BJ. 2000. Age-related changes in photosynthesis of woody plants. *Trends in Plant Science* 5: 349-353.

Bréda N, Huc R, Granier A, Dreyer E. 2006. Temperate forest trees and stands under severe drought: a review of ecophysiological responses, adaptation processes and long-term consequences. *Annals of Forest Science* 63: 625-644.

Breed MF, Marklund MH, Ottewell KM, Gardner MG, Harris JBC, Lowe AJ. 2012. Pollen diversity matters: revealing the neglected effect of pollen diversity on fitness in fragmented landscapes. *Molecular Ecology* 21(24): 5955-5968.

Breed M, Stead M, Ottewell K, Gardner M, Lowe A. 2013a. Which provenance and where? Seed sourcing strategies for revegetation in a changing environment. *Conservation Genetics* 14: 1-10.

Breed MF, Ottewell KM, Gardner MG, Marklund MHK, Dormontt EE, Lowe AJ. 2013b. Mating patterns and pollinator mobility are critical traits in forest fragmentation genetics. *Heredity* doi:10.1038/hdy.2013.48.

Brendel O, Le Thiec D, Scotti-Saintagne C, Bodénès C, Kremer A, Guehl JM. 2008. Quantitative trait loci controlling water use efficiency and related traits in *Quercus robur* L. *Tree Genetics & Genomes* 4: 263-278.

Broadhurst LM, Lowe A, Coates DJ, Cunningham SA, McDonald M., Vesk PA, Yates C. 2008. Seed supply for broadscale restoration: maximizing evolutionary potential. *Evolutionary Applications* 1: 587-597.

Bürgi M, Schuler A. 2003. Driving forces of forest management - an analysis of regeneration practices in the forests of the Swiss Central Plateau during the 19th and 20th century. *Forest Ecology and Management* 176: 173-183.

Burnham KP, Anderson DR. 2001. Kullback-Leibler information as a basis for strong inference in ecological studies. *Wildlife Research* 28: 111-119.

Bush RM, Smouse PE. 1991. The impact of electrophoretic genotype on life history traits in *Pinus taeda*. *Evolution* 45: 481-498.

Bush RM, Smouse PE, Ledig FT. 1987. The fitness consequences of multiple-locus heterozygosity: the relationship between heterozygosity and growth rate in pitch pine (*Pinus rigida* Mill.). *Evolution* 41: 787-798.

Buschbom J, Yanbaev Y, Degen B. 2011. Efficient long-distance gene flow into an isolated relict oak stand. *Journal of Heredity* 102: 464-472.

Butcher PA, Skinner AK, Gardiner CA. 2005. Increased inbreeding and interspecies gene flow in remnant populations of the rare *Eucalyptus benthamii*. *Conservation Genetics* 6: 213-226.

Byrne M, Elliott CP, Yates C, Coates DJ. 2007. Extensive pollen dispersal in a bird-pollinated shrub, *Calothamnus quadrifidus*, in a fragmented landscape. *Molecular Ecology* 16: 1303-1316.

Cavender-Bares J, Bazzaz FA. 2000. Changes in drought response strategies with ontogeny in *Quercus rubra*: implications for scaling from seedlings to mature trees. *Oecologia* 124: 8-18.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009. A quantitative review of heterozygosity-fitness correlations in animal populations. *Molecular Ecology* 18: 2746-2765.

Chazdon R. 2008. Beyond deforestation: restoring forests and ecosystem services on degraded lands. *Science* 320: 1458-1459.

Chen ZD, Manchester SR, Sun HY. 1999. Phylogeny and evolution of the Betulaceae as inferred from DNA sequences, morphology and paleobotany. *American Journal of Botany* 86: 1168-1181.

Chybicki IJ, Burczyk J. 2010. Realized gene flow within mixed stands of *Quercus robur* L. and *Q. petraea* (Matt.) L. revealed at the stage of naturally established seedling. *Molecular Ecology* 19: 2137-2151.

Coart E, De Vreese R, De Cuyper B. 2001. Studie van Vlaamse relictbestanden van inlandse eik: Genetische diversiteit en situering in Europese migratiestromen. VLINA 00/09, Ministry of the Flemish Community, Brussels.

Coates DJ, Hamley VL. 1999. Genetic divergence and the mating system in the endangered and geographically restricted species, *Lambertia orbifolia* Gardner (Proteaceae). *Heredity* 83: 418-427.

Cochard H, Bréda N, Granier A. 1996. Whole tree hydraulic conductance and water loss regulation in *Quercus* during drought: evidence for stomatal control of embolism? *Annales des Sciences Forestières* 53: 197-206.

Cockerham CC. 1969. Variance of gene frequencies. *Evolution* 23: 72-84.

Coltman DW, Slate J. 2003. Microsatellite measures of inbreeding: a meta-analysis. *Evolution* 57: 971-983.

Coulson TN, Pemberton JM, Albon SD, Beaumont MT, Marshall C, Slate J, Guinness FE, Clutton-Brock TH. 1998. Microsatellites reveal heterosis in red deer. *Proceedings of the Royal Society of London B: Biological Sciences* 265: 489-495.

Cousens RD, Rawlinson AA. 2001. When will plant morphology affect the shape of a seed dispersal 'kernel'? *Journal of Theoretical Biology* 211: 229-238.

Craft KJ, Ashley MV. 2010. Pollen-mediated gene flow in isolated and continuous stands of bur oak, *Quercus macrocarpa* (Fagaceae). *American journal of botany* 97(12): 1999-2006.

Crayn DM, Rosetto M, Maynard DJ. 2006. Molecular phylogeny and dating reveals an Oligo-Miocene radiation of dry-adapted shrubs (Tremandraceae) from rain forest tree progenitors (Elaeocarpaceae) in Australia. *American Journal of Botany* 93: 1328-1342.

Crow JF, Kimura M. 1970. An introduction to population genetics theory. Harper & Row, New York.

Dayanandan S, Dole J, Bawa K, Kesseli R. 1999. Population structure delineated with microsatellite markers in fragmented populations of a tropical tree, *Carapa guianensis* (Meliaceae). *Molecular Ecology* 10: 1585-1592.

David P. 1998. Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* 80: 531-537.

David P, Jarne P. 1997. Context-dependent survival differences among electrophoretic genotypes in natural populations of the marine bivalve *Spisula ovalis*. *Genetics* 146: 335-344.

Didham RK., Ghazoul J, Stork NE, Davis AJ. 1996. Insects in fragmented forests; a functional approach. *Trends in Ecology & Evolution* 11: 255-260.

Domec JC,Warren JM, Meinzer FC, Brooks JR, Coulombe R. 2004. Native root xylem embolism and stomatal closure in stands of Douglas-fir and ponderosa pine: mitigation by hydraulic redistribution. *Oecologia* 141: 7-16.

Donovan L, Ehleringer JR. 1991. Ecophysiological differences among juvenile and reproductive plants of several woody species. *Oecologia* 86: 594-597.

Dostálek J, Frantík T, Lukášová M. 2011. Genetic differences within natural and planted stands of *Quercus petraea*. *Central European Journal of Biology* 6: 597-605.

Dow BD, Ashley MV, Howe HF. 1995. Characterization of highly variable (GA/CT) microsatellites in the bur oak, *Quercus macrocarpa*. *Theoretical and Applied Genetics* 91: 137-141.

Dow BD, Ashley MV. 1996. Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa*. *Molecular Ecology* 5: 615-627.

Dow BD, Ashley MV. 1998. High levels of gene flow in bur oak revealed by paternity analysis using microsatellites. *Journal of Heredity* 89: 62-70.

Ducousso A, Michaud H, Lumaret R. 1993. Reproduction and gene flow in the genus *Quercus*. *Annales des Sciences Forestières* 502: 91-106.

Dyer RJ, Sork VL. 2001. Pollen pool heterogeneity in shortleaf pine, *Pinus echinata* Mill. *Molecular Ecology* 10: 859-866.

Eckert CG, Kalisz S, Geber MA, Sargent R, Elle E, Cheptou PO, Goodwillie C, Johnston MO, Kelly JK, Moeller DA, Porcher E, Ree RH, Vallejo-Marín M, Winn AA. 2010. Plant mating systems in a changing world. *Trends in Ecology and Evolution* 25: 35-43.

Edmands S. 2007. Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. *Molecular Ecology* 16(3): 463-475.

Ehrlén J, Lehtilä K. 2002. How perennial are perennial plants? *Oikos* 98: 308-22.

El-Kassaby YA, Dunsworth BG, Krakowski J. 2003. Genetic evaluation of alternative silvicultural systems in coastal montane forests: western hemlock and amabilis fir. *Theoretical and Applied Genetics* 107: 598-610.

Ellstrand NC. 1992. Gene flow by pollen: implications for plant conservation genetics. *Oikos* 63: 77-86.

Ellstrand NC, Elam DR. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics* 24: 217-242.

El Mousadik A, Petit RJ. 1996. Chloroplast DNA phylogeography of the argan tree of Morocco. *Molecular Ecology* 5: 547-555.

England PR, Cornuet J-M, Berthier P, Tallmon DA, Luikart G. 2006. Estimating effective population size from linkage disequilibrium: severe bias in small samples. *Conservation Genetics* 7: 303-308.

Epron D, Dreyer E. 1993. Photosynthesis of oak leaves underwater stress: maintenance of high photochemical efficiency of photosystem II and occurrence of non-uniform CO^2 assimilation. *Tree Physiology* 13: 107-117.

Erikson DL, Fenster CB. 2006. Intraspecific hybridization and the recovery of fitness in the native legume *Chamaecrista fasciculata*. *Evolution* 60: 225-233.

Evans GC. 1972. The quantitative analysis of plant growth. University of California Press, Berkeley.

Ewers RM, Didham RK. 2006. Confounding factors in the detection of species responses to habitat fragmentation. *Biological Reviews* 81(1): 117-142.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131(2): 479-491.

Excoffier L, Hofer T, Foll M. 2009. Detecting loci under selection in a hierarchically structured population. *Heredity* 103(4): 285-298.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Ezard THG, Travis JMJ. 2006. The impact of habitat loss and fragmentation on genetic drift and fixation time. *Oikos* 114: 367-375.

FAO. 2010. Global Forest Resources Assessment 2010 - main report. FAO Forestry Paper (163), Rome.

Farrell EP, Führer E, Ryan D, Andersson F, Hüttl R, Piussi P. 2000. European forest ecosystems: building the future on the legacy of the past. *Forest Ecology and Management* 132(1): 5-20.

Farwig N, Böhning-Gaese K, Bleher B. 2006. Enhanced seed dispersal of *Prunus africana* in fragmented and disturbed forests? *Oecologia* 147: 238-252.

Farwig N, Braun C, Böhning-Gaese K. 2008. Human disturbance reduces genetic diversity of an endangered tropical tree, *Prunus Africana* (Rosaceae). *Conservation Genetics* 9: 317-326.

Fedorov S. 2008. GetData graph digitizer. Version 2.24. GetData, Kogarah, Australia.

Fernández-Manjarrés JF, Sork VL. 2005. Mating patterns of a subdivided population of the Andean oak (*Quercus humboldtii* Bonpl., Fagaceae). *Journal of Heredity* 96: 635-643.

Fernández-Manjarrés JF, Idol J, Sork VL. 2006. Mating patterns of black oak *Quercus velutina* (Fagaceae) in a Missouri Oak-Hickory Forest. *Journal of Heredity* 97: 451-455.

Field AP. 2001. Meta-analysis of correlation coefficients: a Monte Carlo comparison of fixed- and random-effects methods. *Psychological Methods* 6: 161-180.

Finkeldey R, Ziehe M. 2004. Genetic implications of silvicultural regimes. *Forest Ecology and Management* 197: 231-244.

Forest Europe, Unece, FAO. 2011. State of Europe's Forests 2011. Status and trends in sustainable forest management in Europe. Ministerial conference on the protection of forests in Europe, Forest Europe Liaison Unit Oslo, Aas, Norway.

Fort C, Fauveau ML, Muller F, Label P, Granier A, Dreyer E. 1997. Stomatal conductance, growth and root signaling in young oak seedlings subjected to partial soil drying. *Tree Physiology* 17: 281-289.

Fox CW, Reed DH. 2011. Inbreeding depression increases with environmental stress an experimental study and meta-analysis. *Evolution* 65(1): 246-258.

Fox CW, Stillwell RC, Wallin WG, Curtis CL, Reed DH. 2011. Inbreeding-environment interactions for fitness: complex relationships between inbreeding depression and temperature stress in a seed-feeding beetle. *Evolutionary ecology* 25(1): 25-43.

Frankham R, Brook BW, Bradshaw CJ, Traill LW, Spielman D. 2013. 50/500 rule and minimum viable populations: response to Jamieson and Allendorf. *Trends in Ecology and Evolution* 28: 187-188.

Frankham R, Bradshaw CJ, Brook BW. 2014. Genetics in conservation management: revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation* 170: 56-63.

Franklin IR. 1980. Evolutionary change in small populations. In: Soule ME, Wilcox BA. (ed) *Conservation Biology an Evolutionary-Ecological Perspective*. Sinauer Associates, Sunderland, pp 135-150.

Franklin IR, Frankham R. 1998. How large must populations be to retain evolutionary potential? *Animal Conservation* 1: 69-73.

Gagné P, Dayton CM. 2002. Best regression model using information criteria. *Journal of Modern Applied Statistical Methods* 1: 57.

Gailing O, Langenfeld-Heyser R, Polle A, Finkeldey R. 2008. Quantitative trait loci affecting stomatal density and growth in a *Quercus robur* progeny: implications for the adaptation to changing environments. *Global Change Biology* 14: 1934-1946.

Gauzere J, Klein EK, Oddou-Muratorio S. 2013. Ecological determinants of mating system within and between three *Fagus sylvatica* populations along an elevational gradient. *Molecular ecology* 22(19): 5001-5015.

Gernandt DS, Geada Lopez G, Garcia SO, Liston A. 2005. Phylogeny and classification of *Pinus*. *Taxon* 54: 29-42.

Ghazoul J. 2005. Pollen and seed dispersal among dispersed plants. *Biological Reviews*: 80: 413-433.

Gitzendanner MA, Soltis PS. 2000. Patterns of genetic variation in rare and widespread congeners. *American Journal of Botany* 87: 783-792.

González-Varo JP, Aparicio A, Lavergne S, Arroyo J, Albaladejo RG. 2012. Contrasting heterozygosity-fitness correlations between populations of a self-compatible shrub in a fragmented landscape. *Genetica* 140: 31-38.

Gómez JM. 2003. Spatial patterns in long-distance dispersal of *Quercus ilex* acorns by jays in an heterogeneous landscape. *Ecography* 26: 573-584.

Götmark F. 2007. Careful partial harvesting in conservation stands and retention of large oaks favor oak regeneration. *Biological Conservation* 140: 349-358.

Goubitz S, Werger MJ, Shmida A, Ne'eman G. 2002. Cone abortion in *Pinus halepensis*: the role of pollen quantity, tree size and cone location. *Oikos* 97(1): 125-133.

Goudet J. 1995. FSTAT version 1.2: a computer program to calculate F-statistics. *Journal of Heredity* 86: 485-486.

Goudet J, Keller L. 2002. The correlation between inbreeding and fitness: does allele size matter? *Trends in Ecology & Evolution* 17: 201-202.

Goverde M, Schweizer K, Baur B, Erhardt A. 2002. Small-scale habitat fragmentation effects on pollinator behaviour: experimental evidence from the bumblebee *Bombus veterans* on calcareous grasslands. *Biological Conservation* 104: 293-299.

Grafen A. 1989. The phylogenetic regression. *Philosophical Transactions of the Royal Society of London* 205: 581-598.

Granier A, Reichstein M, Bréda N, et al. 2007. Evidence for soil water control on carbon and water dynamics in European forests during the extremely dry year 2003. *Agricultural and Forest Meteorology* 143: 123-145.

Gram WK, Sork VL. 2001. Association between environmental and genetic heterogeneity in forest tree populations. *Ecology* 82: 2012-2021.

Greenberg CH. 2000. Individual variation in acorn production by five species of southern Appalachian oaks. *Forest Ecology and Management* 132: 199-210.

Gurevitch J, Hedges LV. 2001. Meta-analysis: combining the results of independent experiments. In: Scheiner SM, Gurevitch J. (ed) *Design and analysis of ecological experiments*. 2nd edition, Oxford University Press, New York, pp 347-369.

Hampe A, Petit RJ. 2005. Conserving biodiversity under climate change: the rear edge matters. *Ecology Letters* 8: 461-467.

Hampe A, El Masri L, Petit RJ. 2010. Origin of spatial genetic structure in an expanding oak population. *Molecular Ecology* 19: 459-471.

Hamrick JL. 2004. Response of forest trees to global environmental changes. *Forest Ecology Management* 197: 323-335.

Hamrick JL, Godt MJW, Sherman-Broyles SL. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* 6: 95-124.

Hamrick JL, Murawski DA, Nason JD. 1993. The influence of seed dispersal mechanisms on the genetic structure of tropical tree populations. In: Fleming TH, Estrada A. (ed) *Frugivory and Seed Dispersal: Ecological and Evolutionary Aspects*. Kluwer, Dordrecht, pp 281-298.

Hansson B, Westerberg L. 2002. On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology* 11: 2467-2474.

Hardy OJ, Vekemans X. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* 2: 618.

Hardy OJ, Vekemans X. 2003. SPAGeDi 1.1. A program for spatial pattern analysis of genetic diversity. User's manual <http://www.ulb.ac.be/sciences/lagev/fichiers/manual SPAGeDi_1-1.pdf>.

Hardy OJ, González-Martínez SC, Colas B, Fréville H, Mignot A, Olivieri I. 2004. Fine-scale genetic structure and gene dispersal in *Centaurea corymbosa* (Asteraceae). II. Correlated paternity within and among sibships. *Genetics* 168(3): 1601-1614.

Hardy OJ, Maggia L, Bandou E, Breyne P, Caron H, Chevallier MH, Doligez A, Dutech C, Kremer A, Latouche-Hallé C, Troispoux V, Veron V, Degen B. 2006. Fine-scale

genetic structure and gene dispersal inferences in 10 Neotropical tree species. *Molecular Ecology* 15(2): 559-571.

Hedges LV, Olkin I. 1985. Statistical methods for meta-analysis. Academic Press, San Diego, California.

Hedrick PW, Kalinowski ST. 2000. Inbreeding depression in conservation biology. *Annual Review of Ecology and Systematics* 31: 139-162.

Hedrick PW, Fredrickson R, Ellegren H. 2001. Evaluation of d^2, a microsatellite measure of inbreeding and outbreeding, in wolves with a known pedigree. *Evolution* 55: 1256-1260.

Hedrick P. 2005. Large variance in reproductive success and the *Ne*/*N* ratio. *Evolution* 59(7): 1596-1599.

Hemery GE, Savill PS, Pryor SN. 2005. Applications of the crown diameter-stem diameter relationship for different species of broadleaved trees. *Forest Ecology and Management* 215: 285-294.

Hermy M, Verheyen K. 2007. Legacies of the past in the present-day forest biodiversity: a review of past land-use effects on forest plant species composition and diversity *Ecology Resources* 22: 361-371.

Hoebee SE, Arnold U, Düggelin C, Gugerli F, Brodbeck S, Rotach P, Holderegger R. 2007. Mating patterns and contemporary geneflow by pollen in a large continuous and a small isolated population of the scattered forest tree *Sorbus torminalis*. *Heredity* 99: 47-55.

Honig MA, Linder HP, Bond WJ. 1992. Efficacy of wind pollination: pollen load size and natural microgametophyte population in wind-pollinated *Staberoha banksii* (Restionaceae). *American Journal of Botany* 79: 443-448.

Honnay O, Coart E, Butaye J, Adriaens D, Roldan-Ruiz I. 2006. Low effects of historical landscape configuration on population genetic structure of *Anthyllis vulneraria*. *Biological Conservation* 27: 411-419.

Honnay O, Jacquemyn H. 2007. Susceptibility of common and rare plant species to the genetic consequences of habitat fragmentation. *Conservation Biology* 21: 823-831.

Honnay O, Bossuyt B, Jacquemyn H, Shimono A, Uchiyama K. 2008. Can a seed bank maintain the genetic variation in the above ground plant population? *Oikos* 117: 1-5.

Houle D. 1998. How should we explain variation in the genetic variance of traits? *Genetica* 102(3): 241-253.

Hufford KM, Hamrick JL. 2003. Variability selection at three early life stages of the tropical tree *Platypodium elegans* (Fabaceae, Papilonoidae). *Evolution* 57: 518-526.

Husband BC, Schemske DW. 1996. Evolution of the magnitude of inbreeding depression in plants. *Evolution* 50: 54-70.

Hyttinen P. 2001. Prospects for small-scale forestry in Europe. In: Niskanen A, Väyrynen J. (Ed) *Economic sustainability of small-scale forestry*. EFI Proceedings, Finland, pp 21-27.

Ismail SA, Ghazoul J, Ravikanth G, Uma Shaanker R, Kushalappa CG, Kettle CJ. 2012. Does long-distance pollen dispersal preclude inbreeding in tropical trees? Fragmentation genetics of *Dysoxylum malabaricum* in an agro-forest landscape. *Molecular Ecology* 21(22): 5484-5496.

Jacquemyn H, Brys R, Vandepitte K, Honnay O, Roldán-Ruiz I. 2006. Fine-scale genetic structure of life history stages in the food-deceptive orchid *Orchis purpurea*. *Molecular Ecology* 15: 2801-2808.

Jacques D, De Cuyper B. 1998. Genetic conservation strategy for social broadleaves in Belgium. In: report of the first EUFORGEN meeting on social broadleaves, Bordeaux (France).

Jump AS, Peñuelas J. 2005. Running to stand still: adaptation and the response of plants to rapid climate change. *Ecology Letters* 8: 1010-1020.

Jump AS, Peñuelas J. 2006. Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. *Proceedings of the National Academy of Sciences of the United States of America* 103: 8096-8100.

Jump AS, Marchant R, Peñuelas J. 2009. Environmental change and the option value of genetic diversity. *Trends in Plant Science* 14: 51-58.

Kamm U, Rotach P, Gugerli F, Siroky M, Edwards P, Holderegger R. 2009. Frequent long-distance gene flow in a rare temperate forest tree (*Sorbus domestica*) at the landscape scale. *Heredity* 103(6): 476-482.

Kampfer S, Lexer C, Glössl J, Steinkellner H. 1998. Characterization of (GA) microsatellite loci from *Quercus robur*. *Hereditas* 129: 183-186.

Kaufmann SR, Smouse PE, Alvarez-Buylla ER. 1998. Pollen mediated gene flow and differential male reproductive success in a tropical pioneer tree, *Cecropia obtusifolia* Bertol. (Moraceae.). *Heredity* 81: 164-173.

Keller LF, Waller DM. 2002. Inbreeding effects in wild populations. *Trends in Ecology & Evolution* 17: 230-241.

Kettle CJ, Hollingsworth PM, Jaffre T, Moran B, Ennos RA. 2007. Identifying the early genetic consequences of habitat degradation in a highly threatened tropical conifer, *Araucaria nemorosa* de Laubenfels. *Molecular Ecology* 16: 3581-3591.

Kimura M, Ohta T. 1969. The average number of generations until fixation of a mutant gene in a finite population. *Genetics* 61(3): 763-771.

King D. 1981. Tree dimensions: maximizing the rate of height growth in dense stands. *Oecologia* 51: 351-356.

Kirschbaum MUF. 2000. Forest growth and species distribution in a changing climate. *Tree Physiology* 20: 309-322.

Klekowski EJ Jr. 1988. Genetic load and its causes in long-lived plants. *Trees* 2: 195-203.

Knapp EE, Goedde MA, Rice KJ. 2001. Pollen-limited reproduction in blue oak: implications for wind pollination in fragmented populations. *Oecologia* 128: 48-55.

Kramer AT, Ison JL, Ashley MV, Howe HF. 2008. The paradox of forest fragmentation genetics. *Conservation Biology* 22: 878-885.

Kramer A, Havens K. 2009. Plant conservation genetics in a changing world. *Trends in Plant Science* 14: 599-607.

Kramer AT, Fant JB, Ashley MV. 2011. Influences of landscape and pollinators on population genetic structure: examples from three *Penstemon* (Plantaginaceae) species in the Great Basin. *American Journal of Botany* 98: 109-121.

Kremer A, Petit RJ. 1993. Gene diversity in natural populations of oak species. *Annals of Forest Science* 50: 186-202.

Kuussaari M, Bommarco R, Heikkinen RK, Helm A, Krauss J, Lindborg R, Ockinger E, Partel M, Pino J, Roda F, Stefanescu C, Teder T, Zobel M, Steffan-Dewenter I. 2009. Extinction debt: a challenge for biodiversity conservation. *Trends in Ecology & Evolution* 24: 564-571.

Lacy RC. 1987. Loss of genetic diversity from managed populations: Interacting effects of drift, mutation, immigration, selection, and population subdivision. *Conservation Biology* 1: 143-158.

Lajeunesse MJ. 2009. Meta-analysis and the comparative phylogenetic method. *The American Naturalist* 174: 369-381.

Lande R. 1988. Genetics and demography in biological conservation. *Science* 241: 1455-1460.

Lande R. 1995. Breeding plans for small populations based on the dynamics of quantitative genetic variance. In: Ballou JD, Gilpin M, Foose TJ. (ed) *Population management for survival & recovery*. Columbia University Press, New York, pp 318-340.

Ledig FT, Guries RP, Bonefeld BA. 1983. The relation of growth to heterozygosity in pitch pine. *Evolution* 37: 1227-1238.

Lefèvre F. 2004. Human impacts on forest genetic resources in the temperate zone: an updated review. *Forest Ecology and Management* 197: 257-271.

Lemée G. 1987. Les populations de chênes (*Quercus petraea* Liebl.) des réserves biologiques de La Tillaie et du Gros Fouteau en forêt de Fontainebleau: structure, démographie et évolution. *Revue d'Ecologie* 42: 329-355.

Lesbarrères D, Primmer SR, Laurila A, Merilä J. 2005. Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. *Molecular Ecology* 14: 311-323.

Lesica P, Allendorf FW. 1992. Are small populations of plants worth preserving? *Conservation Biology* 6(1): 135-139.

Levin DA, Kerster HW. 1974. Gene flow in seed plants. *Evolutionary Biology* 7: 139-220.

Li RQ, Chen ZD, Lu AM, Soltis DE, Soltis PS, Manos PS. 2004. Phylogenetic relationships in *Fagales* based on DNA sequences from three genomes. *International Journal of Plant Sciences* 165: 311-324.

Light RJ, Pillemer DB. 1984. Summing up: the science of reviewing research. *Proceedings of the Royal Society B* 271: 1961-1966.

Lindenmayer DB, Margules CR, Botkin DB. 1999. Indicators of biodiversity for ecologically sustainable forest management. *Conservation Biology* 14: 941-950.

Lindenmayer DB, Franklin JF. 2002. Conserving forest biodiversity: a comprehensive multi-scaled approach. Island Press, Washington.

Lindner M, Maroschek M, Netherer S, Kremer A, Barbati A, Garcia-Gonzalo J, Seidl R, Delzon S, Corona P, Kolström M, Lexer MJ, Marchetti M. 2010. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. *Forest Ecology and Management* 259: 698-709.

Lipsey MW, Wilson DB. 2001. Practical meta-analysis. Sage Publications, Thousand Oaks, California.

Llorens TM, Ayre DJ, Whelan RJ. 2004. Evidence for ancient genetic subdivision among recently fragmented populations of the endangered shrub *Grevillea caleyi* (Proteaceae). *Heredity* 92: 519-526.

Llorens TM, Byrne M, Yates CJ, Nistelberger HM, Coates DJ. 2012. Evaluating the influence of different aspects of habitat fragmentation on mating patterns and pollen dispersal in the bird-pollinated *Banksia sphaerocarpa* var. caesia. *Molecular Ecology* 21(2): 314-328.

Loiselle BA, Sork VL, Nason J, Graham C. 1995. Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *American Journal of Botany* 82: 1420-1425.

Lonsdale D, Pautasso M, Holdenrieder O. 2008. Wood-decaying fungi in the forest: conservation needs and management options. *European Journal of Forest Research* 127(1): 1-22.

López-Pujol J, Zhang FM, Ge S. 2008. No correlation between heterozygosity and vegetative fitness in the narrow endemic and critically endangered *Clematis acerifolia* (Ranunculaceae). *Biochemical genetics* 46(7-8): 433-445.

Lorimer CG. 1992. Causes of the oak regeneration problem. In: Loftis DL, McGee CE. (ed) *Oak regeneration: serious problems, practical recommendations*. General Technical Report SE-84, US Forest Service, Washington. pp 14-39.

Lowe AJ, Harris S, Ashton P. 2004. Ecological genetics: design, analysis, and application. Blackwell Publishing, Oxford, United Kingdom.

Lowe AJ, Boshier D, Ward M, Bacles CFE, Navarro C. 2005. Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. *Heredity* 95: 255-273.

Lynch M. 1991. The genetic interpretation of inbreeding and outbreeding depression. *Evolution* 45: 622-629.

Maddison WP, Maddison DR. 2009. Mesquite: a modular system for evolutionary analysis. Version 2.72. Available from http://mesquiteproject.org (accessed April 7, 2010).

Maes WH, Fontaine M, Rongé K, Hermy M, Muys B. 2011. A quantitative indicator framework for stand level evaluation and monitoring of environmentally sustainable forest management. *Ecological Indicators* 11: 468-479.

Manly BFJ. 1985. The statistics of natural selection. Chapman and Hall, London.

Manzanera JA, Astorga R, Bueno MA. 1993. Somatic embryo induction and germination in *Quercus suber* L. *Silvae Genetica* 42: 90-93.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* 7: 639-655.

Mariette S, Cottrell J, Csaikl UM, Goikoechea P, König A, Lowe AJ, Van Dam BC, Barreneche T, Bodénès C, Streiff R, Burg K, Groppe K, Munro RC, Tabbener H, Kremer A. 2002. Comparison of levels of genetic diversity detected with AFLP and microsatellite markers within and among mixed *Q. petraea* (Matt.) Liebl. and *Q. robur* L. stands. *Silvae Genetica* 51: 72-79.

Mast RM, Givnish TJ. 2002. Historical biogeography and the origin of stomatal distributions in *Banksia* and *Dryandra* (Proteaceae) based on their cpDNA phylogeny. *American Journal of Botany* 89: 1311-1323.

Matthies D, Bräuer I, Maibom W, Tscharntke T. 2004. Population size and the risk of local extinction: empirical evidence from rare plants. *Oikos* 105(3): 481-488.

McCree KJ. 1972. Test of current definitions of photosynthetically active radiation against leaf photosynthesis data. *Agricultural Meteorology* 10: 443-453.

McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T, Plaut J, Sperry J, West A, Williams DG, Yepez EA. 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist* 178: 719-739.

Millennium Ecosystem Assessment. 2005. Ecosystems and human wellbeing: current state and trends. Island Press, Washington DC.

Mitton JB. 1993. Enzyme heterozygosity, metabolism, and developmental stability. *Genetica* 89: 47-65.

Mitton JB. 1997. Selection in natural populations. Oxford University press, UK.

Mitton JB, Knowles P, Sturgeon KB, Linhart YB, Davis M. 1981. Associations between heterozygosity and growth rate variables in three western forest trees. In: Conkle MT. (ed) *Proceedings of the Symposium on Isozymes of North American Forest Trees and Forest Insects*. US Forest Service, Washington, pp 27-34.

Montoya D, Zavala MA, Rodríguez MA, Purves DW. 2008. Animal versus wind dispersal and the robustness of tree species to deforestation. *Science* 320: 1502-1504.

Moran EV, Clark JSC. 2011. Estimating seed and pollen movement in a monoecious plant: A hierarchical Bayesian approach integrating genetic and ecological data. *Molecular Ecology* 20: 1248-1262.

Mueller UG, Wolfenbarger LL. 1999. AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution* 14: 389-394.

Muona O, Harju A. 1989. Effective population sizes, genetic variability, and mating system in natural stands and seed orchards of *Pinus sylvestris*. *Silvae Genetica* 38: 221-229.

Murcia C. 1996. Forest fragmentation and the pollination of neotropical plants. In: Schelhas J, Greenberg R. (ed) *Forest patches in tropical landscapes*. Island Press, Washington DC, pp 19-36.

Myers N. 1997. The world's forests and their ecosystem services. In: Daily GC. (ed) *Nature's services: societal dependence on natural ecosystems*. Island Press, Washington DC, pp 215-235.

Nakanishi A, Tomaru N, Yoshimaru H, Manabe T, Yamamoto S. 2005. Interannual genetic heterogeneity of pollen pools accepted by *Quercus salicina* individuals. *Molecular Ecology* 14: 4469-4478.

Nardini A, Pitt F. 1999. Drought resistance of *Quercus pubescens* as a function of root hydraulic conductance, xylem embolism and hydraulic architecture. *New Phytologist* 143: 485-493.

Nason JD, Hamrick JL. 1997. Reproductive and genetic consequences of forest fragmentation: two case studies of Neotropical canopy trees. *Journal of Heredity* 88: 264-276.

Nathan R, Horn HS, Chave J, Levin SA. 2002. Mechanistic models for tree seed dispersal by wind in dense forests and open landscapes. In: Levey DJ, Silva WR, Galetti M. (ed) *Seed dispersal and frugivory: ecology, evolution and conservation*, CABI International, New York, pp 69-82.

Noss RF. 2001. Beyond Kyoto: forest management in a time of rapid climate change. *Conservation Biology* 15: 578-590.

O'Connell LM, Mosseler A, Rajora OP. 2006. Impacts of forest fragmentation on the mating system and genetic diversity of white spruce (*Picea glauca*) at the landscape level. *Heredity* 97(6): 418-426.

O'Connell LM, Mosseler A, Rajora OP. 2007. Extensive long-distance pollen dispersal in a fragmented landscape maintains genetic diversity in white spruce. *Journal of Heredity* 98(7): 640-645.

Okubo A, Levin SA. 1989. A theoretical framework for data analysis of wind dispersal of seed and pollen. *Ecology* 70: 329-338.

Oostermeijer JGB, van Eijck MW, den Nijs JCM. 1994. Offspring fitness in relation to population size and genetic variation in the rare perennial plant species *Gentiana pneumonanthe* (Gentianaceae). *Oecologia* 97: 289-296.

Oren R, Pataki DE. 2001. Transpiration in response to variation in microclimate and soil moisture in southeastern deciduous forests. *Oecologia* 127: 549-559.

Ouborg NJ, Vergeer P, Mix C. 2006. The rough edges of the conservation genetics paradigm for plants. *Journal of Ecology* 94: 1233-1248.

Ouborg NJ, Pertoldi C, Loeschcke V, Bijlsma R, Hedrick PW. 2010. Conservation genetics in transition to conservation genomics. *Trends in Genetics*: 26: 177-187.

Pagel MD. 1992. A method for the analysis of comparative data. *Journal of Theoretical Biology* 156: 431-442.

Pakkad G, Ueno S, Yoshimaru H. 2008. Gene flow pattern and mating system in a small population of *Quercus semiserrata* Roxb.(Fagaceae). *Forest Ecology and Management* 255(11): 3819-3826.

Palmer AR. 1999. Detecting publication bias in meta-analysis: a case study of fluctuating asymmetry and sexual selection. *The American Naturalist* 154: 220-233.

Parker KC, Hamrick JL, Parker AJ, Nason JD. 2001. Fine-scale genetic structure in *Pinus clausa* (Pinaceae) populations: effects of disturbance history. *Heredity* 87: 99-113.

Pautasso M. 2009. Geographical genetics and the conservation of forest trees. *Perspectives in Plant Ecology, Evolution and Systematics* 11(3): 157-189.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288-295.

Pemberton JM, Coltman DW, Coulson TN, Slate J. 1999. Using microsatellites to measure the fitness consequences of inbreeding and outbreeding. In: Goldstein DB, Schlötterer C. (ed) *Microsatellites: Evolution and Applications*. Oxford University Press, Oxford, pp 151-164.

Petit RJ, Hampe A. 2006. Some evolutionary consequences of being a tree. *Annual Review of Ecology, Evolution, and Systematics* 37: 187-214.

Piotti A, Leonardi S, Buiteveld J, Geburek T, Gerber S, Kramer K, Vettori C, Vendramin GG. 2011. Comparison of pollen gene flow among four European beech (*Fagus sylvatica* L.) populations characterized by different management regimes. *Heredity* 108: 322-331.

Potter D, Eriksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, Kerr M, Robertson KR, Arsenault M, Dickinson TA, Campbell CS. 2007. Phylogeny and classification of Rosaceae. *Plant Systematics and Evolution* 266: 5-43.

ProSilva 1999. Pro Silva Europe Policy. Truttenhausen, France.

Purvis A. 1995. A composite estimate of primate phylogeny. *Philosophical Transactions of the Royal Society of London* 348: 405-421.

Queller DC, Strassmann JE, Hughes CR. 1993. Microsatellites and kinship. *Trends in Ecology & Evolution* 8: 285-288.

Raijmann LE, Van leeuwen NC, Kersten R, Oostermeijer JGB, Den Nijs HCM, Menken SBJ. 1994. Genetic variation and outcrossing rate in relation to population size in *Gentiana pneumonanthe* L. *Conservation Biology* 8: 1014-1026.

Rajora OP, Rahman MH, Buchert GP, Dancik BP. 2000. Microsatellite DNA analysis of genetic effects of harvesting in old-growth eastern white pine (*Pinus strobes*) in Ontario, Canada. *Molecular Ecology* 9: 339-348.

Reed DH, Frankham R. 2003. Correlation between fitness and genetic diversity. *Conservation Biology* 17: 230-237.

Remington DL, O'Malley DO. 2000. Whole-genome characterization of embryonic stage inbreeding depression in a selfed loblolly pine family. *Genetics* 155: 337-348.

Riitters K, Wickham J, O'Neill R, Jones B, Smith E. 2000. Global-scale patterns of forest fragmentation. *Conservation Ecology* 4(2): 3.

Ritland K. 2002. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* 88: 221-228.

Robledo-Arnuncio JJ, Alia R, Gil L. 2004. Increased selfing and correlated paternity in a small population of a predominantly outcrossing conifer, *Pinus sylvestris*. *Molecular Ecology* 13(9): 2567-2577.

Robledo-Arnuncio JJ, Austerlitz F, Smouse PE. 2007. POLDISP: a software package for indirect estimation of contemporary pollen dispersal. *Molecular Ecology Notes* 7: 763-766.

Rosenberg MS, Adams DC, Gurevitch J. 2000. MetaWin: statistical software for meta-analysis. Version 2.1. Sinauer Associates, Sunderland, Massachusetts.

Roussel M, Dreyer E, Montpied P, Le-Provost G, Guehl JM, Brendel O. 2009. The diversity of 13C isotope discrimination in a *Quercus robur* full-sib family is associated with differences in intrinsic water use efficiency, transpiration efficiency, and stomatal conductance. *Journal of Experimental Botany* 60: 2419-2431.

Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* 8(1): 103-106.

Rushton BS. 1976. Pollen grain size in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. *Watsonia* 11(2): 137-140.

Sagnard F, Pichot C, Vendramin GG, Fady B. 2011. Effects of seed dispersal, adult tree and seedling density on the spatial genetic structure of regeneration at fine temporal and spatial scales. *Tree Genetics and Genomes* 7: 37-48.

Sala OE, Chapin III FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, Leemans R, Lodge DM, Mooney HA, Oesterheld M, Poff NL, Sykes MT, Walker BH, Walker M, Wall DH. 2000. Global biodiversity scenarios for the year 2100. *Science* 287: 1770-1774.

Savolainen O, Hedrick P. 1995. Heterozygosity and fitness: no association in Scots pine. *Genetics* 140: 755-766.

Schönenberger J, Anderberg A, Sytsma KJ. 2005. Molecular phylogenetics and patterns of floral evolution in the Ericales. *International Journal of Plant Sciences* 166: 265-288.

Schueler S, Schlünzen KH, Scholz F. 2005. Viability and sunlight sensitivity of oak pollen and its implications for pollen-mediated gene flow. *Trees* 19: 154-161.

Schuster WS, Mitton JB. 2000. Paternity and gene dispersal in limber pine (*Pinus flexilis* James). *Heredity* 84(3): 348-361.

Scotti-Saintagne C, Bodénès C, Barreneche T, Bertocchi E, Plomion C, Kremer A. 2004. Detection of quantitative trait loci controlling bud burst and height growth in *Quercus robur* L. *Theoretical and Applied Genetics* 109: 1648-1659.

Shono K, Cadaweng EA, Durst PB. 2007. Application of assisted natural regeneration to restore degraded tropical forestlands. *Restoration Ecology* 15: 620-626.

Simberloff D. 1999. The role of science in the preservation of forest biodiversity. *Forest Ecology and Management* 115: 101-111.

Sioen G, Roskams P. 2012. Bosvitaliteitsinventaris 2011, Resultaten uit het bosvitaliteitsmeetnet (Level 1). Rapporten van het Instituut voor Natuur- en Bosonderzoek, Instituut voor Natuur- en Bosonderzoek, Brussel.

Slate J, Pemberton JM. 2002. Comparing molecular measures for detecting inbreeding depression. *Journal of Evolutionary Biology* 15: 20-31.

Slate J, David P, Dodds KG, Veenvliet BA, Glass BC, Broad TE, McEwan JC. 2004. Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* 93(3): 255-265.

Smouse PE, Dyer RJ, Westfall RD, Sork VL. 2001. Twogeneration analysis of pollen flow across a landscape. I. Male gamete heterogeneity among females. *Evolution* 55: 260-271.

Smouse PE, Sork VL. 2004. Measuring pollen flow in forest trees: an exposition of alternative approaches. *Forest Ecology and Management* 197: 21-38.

Soltis DE, Soltis PS, Chase MW, Mort ME, Albach DC, Zanis M, Savolainen V, Hahn WH, Hoot SB, Fay MF, Axtell M, Swensen SM, Prince LM, Kress WJ, Nixon KC, Farris JS. 2000. Angiosperm phylogeny inferred from 18S rDNA, rbcL, and atpB sequences. *Botanical Journal of the Linnean Society* 133: 381-461.

Sork VL, Davis FW, Smouse PE, Apsit VJ, Dyer RJ, Fernandez JF, Kuhn B. 2002. Pollen movement in declining populations of California valley oak, *Quercus lobata*: Where have all the fathers gone? *Molecular Ecology* 11: 1657-1668.

Sork VL, Smouse PE. 2006. Genetic analysis of landscape connectivity in tree populations. *Landscape Ecology* 21(6): 821-836.

Sousa VA, Sebbenn AM, Hattemer HH, Ziehe M. 2005. Correlated mating in populations of a dioecious Brazilian conifer, *Araucaria angustifolia* (Bert.) O. Ktze. *Forest Genetics* 12: 107-119.

Stacy EA, Hamrick JL, Nason JD, Hubbell SP, Foster RB, Condit R. 1996. Pollen dispersal in low-density populations of three neotropical tree species. *American Naturalist* 148: 275-298.

Steduto P. 2003. Biomass water-productivity. Comparing the growth-engines of crop models. In: *FAO expert meeting on crop water productivity under deficient water supply*. FAO, Rome.

Steduto P, Hsiao TC, Fereres E. 2007. On the conservative behavior of biomass water productivity. *Irrigation Science* 25: 189-207.

Steinhoff S. 1993. Results of species hybridization with *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. *Annals of Forest Science* 53: 173-180.

Steinkellner H, Fluch S, Turetschek E, Lexer C, Streiff R, Kremer A, Burg K, Glössl J. 1997. Identification and characterization of (GA/CT)n-microsatellite loci from *Quercus petraea*. *Plant Molecular Biology* 33: 1093-1096.

Streiff R, Labbe T, Bacilieri R, Steinkellner H, Gloessl J, Kremer A. 1998. Within-population genetic structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. assessed with isozymes and microsatellites. *Molecular Ecology* 7: 317-328.

Streiff R, Ducousso A, Lexer C, Steinkellner H, Gloessl J, Kremer A. 1999. Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. *Molecular Ecology* 8(5): 831-841.

Stiles FG. 1978. Ecological and evolutionary implications of bird pollination. *American Zoologist* 18: 715-727.

Stocker TF, Qin D, Platner GK, Tignor MMB, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley PM. et al. 2013. Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. UK: Cambridge University Press, Cambridge.

Szulkin M, Bierne N, David P. 2010. Heterozygosity-fitness correlations: a time for reappraisal. *Evolution* 64: 1202-1217.

Tack G, Van den Bremt P, Hermy M. 1993. Bossen van Vlaanderen, een historische ecologie. Davidsfonds, Leuven, Belgium.

Tallmon DA, Luikart G, Waples RS. 2004. The alluring simplicity and complex reality of genetic rescue. *Trends in Ecology & Evolution* 19: 489-496.

Templeton AR. 1986. Coadaptation and outbreeding depression. In Soule ME. (ed) *Conservation Biology The Science of Scarcity and Diversity*, Sinauer Associates, Inc., Sunderland pp 105-116.

Teulat B, Borries C, This D. 2001. New QTLs identified for plant water status, water-soluble carbohydrate and osmotic adjustment in a barley population grown in a growth-chamber under two water regimes. *Theoretical and Applied Genetics* 103: 161-170.

Thompson I, Mackey B, McNulty S, Mosseler S. 2009. Forest resilience, biodiversity, and climate change. A synthesis of the biodiversity/resilience/stability relationship in forest ecosystems. Secretariat of the Convention on Biological Diversity, Montreal.

Vakkari P, Blom A, Rusanen M, Raisio J, Toivonen H. 2006. Genetic variability of fragmented stands of pedunculate oak (*Quercus robur*) in Finland. *Genetica* 127: 231-241.

Valbuena-Carabana M, González-Martínez SC, Sork VL, Collada C, Soto A, Goicoechea PG, Gil L. 2005. Gene flow and hybridisation in a mixed oak forest (*Quercus pyrenaica* Willd. and *Quercus petraea* (Matts.) Liebl.) in central Spain. *Heredity* 95(6): 457-465.

Valladares F, Gianoli E, Gomez JM. 2007. Ecological limits to plant phenotypic plasticity. *New Phytologist* 176: 749-763.

Vamosi JC, Vamosi SM. 2004. The role of diversification in causing the correlates of dioecy. *Evolution* 58: 723-731.

Van Coillie F, Verbeke LP, De Wulf RR. 2007. Feature selection by genetic algorithms in object-based classification of IKONOS imagery for forest mapping in Flanders, Belgium. *Remote Sensing of Environment* 110(4): 476-487.

Vandekerkhove K. 2013. Integration of nature protection in forest policy in Flanders (Belgium). INTEGRATE Country Report, EFICENT-OEF, Freiburg.

Vander Mijnsbrugge K, Bischoff A, Smith B. 2010. A question of origin: where and how to collect seed for ecological restoration. *Basic and Applied Ecology* 11: 300-311.

Van Hees AFM. 1997. Growth and morphology of pedunculate oak (*Quercus robur* L) and beech (*Fagus sylvatica* L) seedlings in relation to shading and drought. *Annales des Sciences Forestières* 54: 9-18.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley PF. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535-538.

Van Rossum F, Bonnin I, Fenart S, Pauwels M, Petit D, Saumitou-Laprade P. 2004. Spatial genetic structure within a metallicolous population of *Arabidopsis halleri*, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* 13(10): 2959-2967.

Vekemans X, Hardy OJ. 2004. New insights from fine scale spatial genetic structure analyses in plant populations. *Molecular Ecology* 13: 921-935.

Vellend M, Verheyen K, Jacquemyn H, Kolb A, Van Calster H, Peterken G, Hermy M. 2006. Extinction debt of forest plants persists for more than a century following habitat fragmentation. *Ecology* 87: 542-548.

Vera FW, Bakker ES, Olff H. 2006. Large herbivores: missing partners of western European light-demanding tree and shrub species? In: Danell K, Bergström R, Duncan P, Pastor J. (ed) *Large Herbivore Ecology, Ecosystem Dynamics and Conservation*, Cambridge University Press, Cambridge, UK, pp 203-231.

Vivin P, Aussenac G, Levy G. 1993. Differences in drought resistance among three deciduous oak species grown in large boxes. *Annales des Sciences Forestières* 50: 221-233.

Vranckx G, Jacquemyn H, Muys B, Honnay O. 2012. Meta-analysis of susceptibility of woody plants to loss of genetic diversity through habitat fragmentation. *Conservation Biology* 26: 228-237.

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Kint V, Muys B, Honnay O. 2014. Transmission of genetic variation from the adult generation to naturally established seedling cohorts in small forest stands of pedunculate oak (*Quercus robur* L.). *Forest Ecology and Management* 312: 19-27.

Wang J. 2004. Application of the one-migrant-per-generation rule to conservation and management. *Conservation Biology* 18(2): 332-343.

Wang H, Sork VL, Wu J, Ge J. 2010. Effect of patch size and isolation on mating patterns and seed production in an urban population of Chinese pine (*Pinus tabulaeformis* Carr.). *Forest Ecology and Management* 260(6): 965-974.

Waples RS, Do C. 2008. LdNe: a program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources* 8: 753-756.

Waterinckx M. 2001. De bosinventarisatie van het Vlaamse Gewest. Deel 2: Bosbouwkundige resultaten. Ministerie van de Vlaamse Gemeenschap, afdeling Bos & Groen.

Weber JA, Gates DM. 1990. Gas exchange in *Quercus rubra* (northern red oak) during a drought: analysis of relations among photosynthesis, transpiration and leaf conductance. *Tree Physiology* 7: 215-225.

Weir BS, Hill WG. 1980. Effect of mating structure on variation in linkage disequilibrium. *Genetics* 95: 477-488.

West P. 2009. Tree and forest measurement. Springer, Heidelberg.

Weston PH, Barker NP. 2006. A new suprageneric classification of the Proteaceae, with an annotated checklist of genera. *Telopea* 11: 314-344.

White GM, Boshier DH, Powell W. 2002. Increased pollen flow counteracts fragmentation in a tropical dry forest: an example from *Swietenia humilis* Zuccarini. *Proceedings of the National Academy of Sciences* 99(4): 2038-2042.

Whitehead DR. 1983. Wind pollination: some ecological and evolutionary perspectives. In Real L. (ed) *Pollination biology*. Academic Press, London, pp 97-108.

Wiersum KF, Elands BHM, Hoogsta MA. 2005. Small-scale forest ownership across Europe: characteristics and future potential. *Small-scale Forest Economics, Management And Policy* 4: 1-9.

Willi Y, Van Buskirk J, Hoffmann AA. 2006. Limits to the adaptive potential of small populations. *Annual Review of Ecology, Evolution and Systematics* 37: 433-458.

Williams M. 2002. Deforesting the earth: from prehistory to global crisis. University of Chicago Press, Chicago, USA.

Williamson-Natesan EG. 2005. Comparisons of methods for detecting bottlenecks from microsatellite loci. *Conservation Genetics* 6: 551-562.

Wilson PG, O'Brien MM, Heslewood MM, Quinn CJ. 2005. Relationships within Myrtaceae sensu lato based on a matK phylogeny. *Plant Systematics and Evolution* 251: 3-19.

Wojciechowski MF, Lavin M, Sanderson MJ. 2004. A phylogeny of legumes (Leguminosae) based on analysis of the plastid matK gene resolves many well-supported subclades within the family. *American Journal of Botany* 91: 1846-1862.

Wright S. 1931. Evolution in Mendelian populations. *Genetics* 16: 97-159.

Wright JP, Jones CG. 2006. The concept of organisms as ecosystem engineers ten years on: progress, limitations, and challenges. *BioScience* 56: 203-209.

Yeh FC, Yang R-C, Boyle T. 1999. POPGENE, version 1.32, the user friendly software for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton.

Young AG, Warwick SI, Merriam HG. 1993. Genetic variation and structure at three spatial scales for *Acer saccharum* (sugar maple) in Canada and implications for conservation. *Canadian Journal of Forest Research* 23: 2568-2578.

Young AG, Boyle T, Brown AHD. 1996. The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution* 11: 413-418.

Young AG, Brown AH. 1999. Paternal bottlenecks in fragmented populations of the grassland daisy *Rutidosis leptorrhynchoides*. *Genetical Research* 73(2): 111-117.

Young AG, Brown AHD, Zich FA. 1999. Genetic structure of fragmented populations of the endangered daisy *Rutidosis leptorrhynchoides*. *Conservation Biology* 13: 256-265.

Ziehe M, Hattemer HH. 2002. Target-diameter felling and consequences for genetic structures in a beech stand (*Fagus sylvatica* L.). In: von Gadow K, Nagel J,

Saborowski J. (ed) *Continuous cover forestry: assessment, analysis, scenarios*. Kluwer Academic Publishers, Dordrecht, pp 91-105.

Zouros E, Foltz DW. 1987. The use of allelic isozyme variation for the study of heterosis. *Isozymes* 15: 1-59.

Zweifel R, Zimmermann L, Newbery DM. 2005. Modeling tree water deficit from microclimate: an approach to quantifying drought stress. *Tree Physiology* 25: 147-156.

Publication List

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Vranckx G, Jacquemyn H, Muys B, Honnay O. 2012. Meta-Analysis of Susceptibility of Woody Plants to Loss of Genetic Diversity through Habitat Fragmentation. *Conservation Biology* 26 (2): 228-237.

Vranckx G, Vandelook F. 2012. A season- and gap-detection mechanism regulates seed germination of two temperate forest pioneers. *Plant Biology* 14: 481-490.

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Kint V, Muys B, Honnay O. 2014. Transmission of genetic variation from the adult generation to naturally established seedling cohorts in small forest stands of pedunculate oak (*Quercus robur*). *Forest Ecology and Management* 312: 19-27.

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Janssens P, Gielen B, Muys B, Honnay O. 2014. The effect of drought stress on heterozygosity-fitness correlations in pedunculate oak (*Quercus robur* L.). *Annals of Botany* 113(6): 1057-1069.

Vranckx G, Mergeay J, Cox K, Muys B, Jacquemyn H, Honnay O. 2014. Tree density and population size affect pollen flow and mating patterns in small fragmented forest stands of pedunculate oak (*Quercus robur* L.). *Forest Ecology and Management* 328: 254-261.